ASSESSMENT OF A CHEMILUMINESCENT DETECTION SYSTEM FOR FORENSIC DNA RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

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An abstract of a Thesis by

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The purpose of this project was to determine whether a chemiluminescent detection system can give the sensitivity of ^{32}P isotopic detection for human DNA restriction fragment length polymorphism (RFLP) analysis and have the speed, safety, and reduced cost of colorimetric detection. The assessment was made on the basis of: cost, sensitivity, time of operation, ease of operation, and ease of multiple probings. Human DNA was extracted from whole blood and processed for RFLP analysis according to the December 1990 FBI Protocol: "Procedures for the Detection of Restriction Fragment Length Polymorphisms in Human DNA." Both the Boehringer Mannheim Genius™ system which uses digoxigenin labeled probes detected with alkaline phosphatase-conjugated anti-digoxigenin antibody, and the Promega Gene Print Light™ system which uses alkaline phosphatase bound directly to the probe, were examined. Probes pH30, YNH24, and TBQ7 were used. Visualization was with Lumi-Phos™ 530 and Kodak XAR X-ray film. Results show that chemiluminescent detection is better than colorimetric or ^{32}P detection in all aspects but sensitivity.

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INTRODUCTION

Since 1987 the use of DNA Restriction Fragment Length Polymorphism (RFLP) for the identification of individuals in forensic case work has increased dramatically. The discriminatory power and the sensitivity of this test have made it the biggest breakthrough for forensic science in decades. With very small samples of biological material such as seminal fluid stains in rape cases or bloodstains in homicide cases, the tests can be used to tie criminals to the crimes.

Current protocols for DNA (RFLP) analyses have classically used ^{32}P detection due to its sensitivity (24); however, recent advances in technology have shown chemiluminescent detection systems hold great promise (11, 21). Current detection procedures include radioisotopic ^{32}P, chemiluminescence, and colorimetric nitroblue tetrazolium salt-5-bromo-4-chloro-3-indoyl phosphate toluidinium salt (NBT-BCIP) (2, 16).

Radioactive detection systems are currently the most sensitive, but have inherent safety drawbacks (19, 24). Colorimetric detection systems are very fast and easy to use but lack the sensitivity of radioactive systems (6). Chemiluminescent detection systems appear to have the best qualities of both systems previously mentioned.

Work during the last three years has shown an expansion of chemiluminescent detection systems by such companies as Tropix (6, 23), Promega, Boehringer Mannheim, GIBCO BRL, and Cellmark (8). Most systems use an alkaline phosphatase enzyme substrate system. The enzyme is usually attached to an antigenic molecule such as biotin, or digoxigenin via an antibody conjugate system (10, 13, 18 ,23). Most recently direct link systems attach the alkaline phosphatase (A-P), directly to the probe DNA (20). Development is performed with photo reactive chemicals such as luminol type dyes with enhancers. The systems use X-ray or Polaroid™ film to record the exposure.

This project was designed to assess chemiluminescent detection of human DNA by RFLP analysis. Assessment was made on the basis of the following: cost, sensitivity, time of operation, ease of operation, ease of reprobing, and safety. Direct comparison with ^{32}P was not possible due to lack of funding and implementation of DNA RFLP by the Iowa Division of Criminal Investigation (DCI) laboratory. The comparison will be made on the basis of personal experience and published results when referring to isotopic detection.

MATERIALS & METHODS

DNA extraction was begun with venupuncture of 21 individuals. Seven ml of whole blood was collected in lavender top EDTA Vacutainer™ (Becton Dickinson, Rutheford, NJ) tubes. Blood was processed by three different protocols. They included the whole blood high salt extraction protocol according to Dykes (9), the organic liquid blood extraction protocol according to the FBI (7), and the organic bloodstain extraction protocol according to the FBI (7). The protocols are as follows:

Dykes whole blood high salt extraction protocol

Seven ml ethylenediaminetetraacetate (EDTA) Vacutainer™ tubes containing whole blood was spun for 20 min at 2000 rpm. The white cell layer (buffy coats) of nucleated cells were removed and placed in 15 ml conical centrifuge tubes. Red blood cell lysis buffer [0.144M ammonium chloride (NH4Cl), 0.001M sodium bicarbonate (NaCHO3)] was added to a final volume of 9 ml. The samples were mixed well and allowed to sit at room temperature for 15 min. The tubes were then spun for 20 min at 2000 rpm and the supernatant was discarded. To the tubes 3 ml of nuclei lysis buffer [10mM Tris, 400mM sodium chloride (NaCl), 2mM sodium EDTA (Na2EDTA) to pH 8.2], was added to the white cell button and agitated to resuspend the cells. Next, 200 ul of 10% sodium dodecyl sulfate (SDS) and 600 ul of proteinase K (2 mg/ml) were then added. The tubes were agitated, and allowed to incubate overnight at 37°C. The next morning 1 ml of saturated 6M sodium chloride (NaCl) was added to the tube, shaken vigorously for 15 sec and spun for 15 min at 2500 rpm. The supernatant containing DNA was transferred to a new 15 ml conical centrifuge tube where approximately 8 ml of room temperature absolute ethanol was added and mixed gently. The precipitated DNA was removed with a 1 ul inoculation loop by spooling it on to the loop and placing it into a

new 1.5 ml microcentrifuge tube. The DNA was resuspended in 200 to 300 ul of Tris EDTA (TE) buffer [10 mM Tris, 0.2 mM Na2EDTA, pH 7.5] at 50°C for 2 hr.

FBI whole blood organic extraction protocol

Seven ml Vacutainer™ tubes of whole blood were mixed well, and divided into 700 ul aliquots. Blood was frozen at -80°C and then thawed. To the thawed blood 800 ul of 1X SSC [0.15M NaCl, 15 mM sodium citrate (Na3C6H5O7) pH 7.0] was added and mixed. The samples were spun in a microcentrifuge for 1 min at 10,000 rpm and the supernatant fluid was removed. The remaining pellet was mixed with 375 ul of 0.2M sodium acetate, 25 ul of 10% SDS and 5 ul of proteinase K (20 mg/ml). This was vortexed and incubated at 56°C for 1 hr. To the DNA 120 ul of phenol/chloroform/isoamyl alcohol was added. The samples were vortexed and centrifuged for 2 min at 10,000 rpm. The aqueous layer was carefully removed and placed in a new 1.5 ml microcentrifuge tube. To the aqueous layer, 1 ml of cold absolute ethanol was added, mixed and centrifuged for 30 sec at 10,000 rpm. The supernatant fluid was removed and an additional wash with 1 ml of 70% ethanol was performed. The samples were centrifuged another 30 sec at 10,000 rpm and the supernatant removed. The DNA pellets were dried in a vacuum centrifuge to remove the remaining ethanol. The pellets were resuspended in 200 ul of (TE) buffer at 56°C overnight.

FBI bloodstain organic extraction protocol

Whole blood was aliquoted on to clean cotton cloth in 100 ul bloodstains and allowed to air dry. The 100 ul bloodstains were cut into small pieces and placed into 1.5 ml microcentrifuge tubes. Added to the sample was 400 ul of stain extraction buffer [10mM Tris, pH 8.0, 10mM EDTA, 0.1M NaCl, 0.039M dithiothreitol (DTT) and 2% SDS] and 10 ul of proteinase K (20 mg/ml). The tubes were vortexed and incubated overnight at 56°C. Small holes were punched into the lids, and the pieces of cut up cloth were placed into the top of the lids. The samples were centrifuged for 5 min at 10,000 rpm and the old cap with

the cuttings was removed. New caps were placed on the tubes and 500 ul of phenol/chloroform/isoamyl alcohol were added to each tube. The tubes were vortexed and centrifuged for 2 min at 10,000 rpm. The aqueous layer was removed and transferred to a new tube. To the aqueous layer, 1ml of cold absolute ethanol was added to each tube and placed at -20°C for 30 min. The precipitated DNA was centrifuged for 15 min at 10,000 rpm and the supernatant was discarded. The DNA pellet was washed with 1 ml of 70% ethanol, centrifuged for 30 sec at 10,000 rpm and the supernatant removed. The DNA pellet was dried in a vacuum centrifuge to remove the remaining ethanol. The DNA was resolubilized in 36 ul of TE buffer at 56°C overnight.

Quantitation

DNA quantitation was performed by U-V visible spectrophotometry according to Maniatis (14), using a Perkin Elmer Lambda 3 U-V visible spectrophotometer with a super sipper. This quantitation was based on the formula that 50 ug DNA/ml demonstrates an A_{260} of 1.0. All liquid blood samples processed by the high salt extraction protocol were quantitated this way.

The remaining DNA samples processed by either of the FBI extraction protocols were quantitated by mini-gel electrophoresis. The mini-gel DNA quantitation protocol (7) is as follows:

FBI Yield gel protocol

Yield (quantitation) gels were performed on Fotodyne mini-gel tanks. Gels were 7.0 cm x 9.2 cm x 0.5 cm thick using 1% Sigma (St. Louis, MO) molecular grade agarose eeo<0.15 in 1X TAE buffer [0.04M Tris., 0.019M Glacial HAc, 0.025M EDTA, pH 8.0]. Tank buffer (1X TAE) was placed in the electrophoresis chamber until the level of the buffer just covered the gel. Both the tank buffer and the gel buffer were spiked with a 5 mg/ml ethidium bromide stock solution for a final working concentration of 0.05 ug/ml. For

each gel six lambda DNA quantitation standards (GIBCO BRL) of: 500, 250, 125, 63, 31 and 15 ng were run. A 23 kb lambda DNA standard, and a known undigested K562 standard cell line were also run on each gel. All standards and samples were loaded with 2 ul of loading dye solution [50% glycerol (w/v), 0.1% bromophenyl blue, and 0.1M EDTA, all in 0.1mM TE buffer]. Electrophoresis was performed for 15 min at 200 V using a Fotodyne model 255 power supply. Visualization of the yield gels was by ultraviolet light at 302 nm. A Fotodyne Polaroid™ backed camera was employed using Polaroid 667 high speed black and white film with a red orange DNA photographic filter #3-4205. An f-stop of f-8 with an exposure time of 1/2 sec was used. Photos were taken for a permanent record. The photograph was used to estimate quantity and quality of high molecular weight DNA in each sample lane by comparing the samples to the quantitation standards, and sample position to the 23 kb lambda DNA standard.

FBI digestion protocol

Digestion with *Hae* III (*Haemophilus aegyptius* III) was performed using a 5-8 fold excess of restriction enzyme. Vendors for the restriction enzyme included: (GIBCO BRL) Bathesda Research Laboratories Life Technologies Inc., (AGTC) Analytical Genetic testing Center, Sigma, and Fisher (Promega). [Note, all restriction enzymes purchased through Fisher are actually received from Promega]. All four vendors' enzymes worked satisfactorily. No star activity was noticed and care was taken to prevent glycerol concentrations from exceeding 10% in the restriction reaction mixture. All digestions with the restriction enzyme *Hae* III were performed using the manufacturers restriction buffers which were included with the enzymes. Reaction volumes for the bloodstain digestions were 32 ul of DNA, 4 ul of restriction buffer concentrate, and 40 Iμ of *Hae* III enzyme (Total volume =40 ul). Reaction volumes for the liquid bloods were 200 ul of DNA, 25 ul of restriction buffer concentrate, x ul of *Hae* III [(x = (6) (ug DNA) (units *Hae* III/ul)], and y ul of water [y = 25-

x](Total volume =250 ul). Incubations all occurred at 37°C overnight.

After digestion DNA samples were reprecipitated. The protocols according to the FBI are as follows:

FBI blood stain reprecipitation protocol

To each 40 ul of DNA digest, 13 ul of 7.0M ammonium acetate (NH4OAc) was added and mixed by hand. Next, 106 ul of cold absolute ethanol was added and mixed again by hand. The samples (tubes) were placed at -20°C for 15-30 min, centrifuged for 15 min at 10,000 rpm and the alcohol decanted off. The pellet was rinsed with 1.0 ml of 70% ethanol, centrifuged another 5 min at 10,000 rpm and the supernatant decanted off. The remaining alcohol was removed by vacuum centrifugation. The sample was resuspended in 16 ul of TE buffer at 56°C for 0-30 min.

FBI liquid blood reprecipitation protocol

To each 250 ul restriction digest, 83 ul of 7.0M (NH4OAc) was added and mixed by hand. Next, 666 ul of absolute ethanol was added and mixed again by hand. The samples were placed at -20°C for 15-30 min, centrifuged for 15 min at 10,000 rpm and the alcohol decanted off. The pellet was rinsed with 1.0 ml of 70% ethanol, centrifuged another 5 min at 10000 rpm and the supernatant decanted off. The remaining alcohol was removed by vacuum centrifugation. The sample was resuspended in 16 ul of TE buffer at 56°C for 30-60 min.

FBI restriction gel protocol

The restriction gel, electrophoresis, and photography parameters were exactly the same as the (FBI yield gel protocol) except for the standards and quantity of sample loaded on each gel. The standards loaded on each restriction gel consisted of 1) 200 ng undigested human K562 sample 2) 200 ng *Hae* III digested human K562 sample and 3) 120 ng of *Hind* III digested lambda DNA. Two microliters of each sample were loaded with 1.0 ul of

loading dye solution on each restriction gel. Completely digested DNA showed as a smooth streak from the dye front back towards the origin.

The final selection of analytical tanks chosen were the GIBCO BRL model H5 11 x 16 cm horizontal submarine tanks. The GIBCO BRL model 500 high current power supply power source was chosen to run the electrophoresis tanks. The analytical gel electrophoresis protocol according to the FBI (7) is as follows:

FBI analytical gel protocol

The analytical gels and buffers were composed of the same concentrations of 1% Sigma molecular grade agarose in 1X TAE buffer, and 0.05 ug/ml ethidium bromide as the yield and restriction gels. Note: When the Gene Print Light™ probes were used, no ethidium bromide was incorporated into the analytical gels or tank buffers. The analytical gel size was 11 x 16 x 0.57 cm thick. The tank buffer covered the gel to a depth of approximately 0.5 cm. Size marker lanes were placed in lanes 6, 10, and 14. The size marker was the Gene Print Light™ Equiladder which contained 29 bands from 485 bp to 25.632 kb and was direct labeled with alkaline phosphatase. An additional marker lane was added in lane 16. This size marker was the GIBCO BRL Photoprobe™ ladder which contained 30 bands from 526 bp to 22.6 kb and was direct labeled with alkaline phosphatase. Voltages were set at 30 V for 17 hrs to produce a run time of approximately 510 volt hrs.

Early on in the project, when the Genius™ kit was used, a Boehringer Mannheim Molecular Marker III (B-M MWM III) size marker was used as a ladder. The ladder was prelabeled with digoxigenin and consisted of lambda DNA cut with both *Eco* RI and *Hind* III. The ladder contained 12 bands from 21.2 kb to 564 bp.

Southern transfer

Southern blotting was performed according to Southern (22) with modifications from the FBI protocol (7) using MSI (Micron Separations Inc. Westborough, MA)

Magnagraph nylon membranes. Three transfer procedures were used. 1) For the Genius™ system a 6 hr alkaline transfer using 0.4N NaOH with thick sponges followed by a 15 min wash in neutralization buffer [0.2M Tris, 2X SSC pH 7.5] was used. 2) For the Genius™ system a high salt transfer was also used. Prior to the 20X SSC [3..0M sodium chloride, 0.3M sodium citrate (Na3 citrate 2H2O) pH 7.0] transfer, the gels were placed for 30 min in denaturing solution [0.5N NaOH, 1.5M NaCl] followed by 30 min in neutralization solution [1.0M Tris, 1.5M NaCl, pH 8.0]. After denaturing and neutralization the Southern transfer took place for 6.0 hr in 20X SSC with thick sponges. 3) For the Promega Gene Print Light™ system a high salt 10X SSC transfer was run. Prior to the transfer, the same denaturing solution [0.5N NaOH, 1.5M NaCl] and neutralization solution [1.0M Tris, 1.5M NaCl pH 8.0] were used to wash the gels for 30 min each. The 10X SSC transfer was run for 5 hrs with thick sponges.

Fixation

After using any of the three Southern transfer procedures, the membranes were placed in equilibration buffer [0.2M Tris, 2X SSC, pH 7.5] for 10 min. Fixation of the DNA to the nylon membranes was performed by both heat baking and U-V cross-linking. The nylon membranes were sandwiched between two pieces of Whatman 3mm paper and vacuum baked at 80°C for 30 min. Following heat baking the nylon membranes were removed from the Whatman paper and placed in a Fisher FBUVXL 1000 and U-V cross-linked for the optimum time setting at 254 nm.

Probes

The probe pH30 or D4S139 was selected. This probe showed that it was a strong binding probe and was very sensitive with ^{32}P detection. Data on the probe showed it was mapped on distal chromosome 4q by multipoint linkage analysis. The maximum lod score 3.01 was obtained with locus CRI-L231. This clone is a 4.5 kb *Sau* 3A fragment in pUC18

subcloned from a EMBL4 clone containing a 20 kb insert in the *Eco*RI site identified with the oligonucleotide H30 (5' -GCCGTGTCCTCGGCTCTCAGG-3')(15). Polymorphism showed greater than 20 VNTR alleles with bands between 4.0 and 25.0 kb with at least 8 restriction enzymes under normal RFLP stringency. The probe has shown Mendelian inheritance in 18 three generation families and 6 two generation families. Quality control on the probe demonstrates that 1 ug of *Hae* III digested K562 DNA was hybridized against the pH30 probe according to the manufacturer's procedure. Only two bands were produced and sized at 6.555 kb and 3.475 kb, which was within ± 2.5% of the accepted allele sizes. Quality control analysis also demonstrated that 400 ng of *Hae* III digested *E. coli* chromosomal DNA was hybridized to probe pH30 with no bands detected.

Probe labeling protocol

Probe labeling was performed using *Hind* III digested lambda phage DNA (Sigma) to optimize the procedure and to prevent wasting the pH30 probe. Labeling the lambda DNA was performed according to the Genius™ kit instructions for random primer labeling. Both a 20 ul and a 50 ul reaction volume were used for an overnight random primer extension reaction. The standard 20 ul reaction mixture included 120 ng of freshly denatured lambda DNA, 2 ul of hexanucleotide mixture, 2 ul of dNTP labeling mixture (including the DIG-dUTP), 1 ul klenow enzyme, and enough double distilled water to make 20 ul. The 50 ul reaction increased each reagent by 2.5 times. All samples were combined while on ice. Random primer extension proceeded overnight for 16-20 hrs at 37°C. The reaction was quenched with 2 ul of 0.2M EDTA [pH 8.0] and precipitated with 1 ul of 20 mg/ml glycogen carrier and 1/10 vol of 1.0M NaCl. Three vol of -20°C absolute ethanol were added to the reaction mixture and incubated at -20°C for 2 hrs. The mixture was centrifuged at 10,000 rpm for 15 min, and the ethanol was decanted off. A second wash of three volumes of 70% ethanol was added to the precipitate, centrifuged at 10,000 rpm for 15 min, and

decanted off. The remaining ethanol was removed by vacuum centrifugation. The dry pellet was resuspended in 50 ul of TE buffer at 37°C for 10 min with frequent vortexing.

Probe quantitation

After labeling and precipitation, the probe was quantitated by a dot blot procedure according to the Genius™ kit instructions. A digoxigenin prelabeled pBR328 DNA source supplied with the kit was run in tandem to the newly labeled probe. Serial dilutions from 1.0 ng to 0.1 pg were made of the control pBR328 and compared to the same serial dilutions of the newly labeled probe. DNA volumes of 1 ul were spotted down from each serial dilution on a nylon membrane and vacuum baked at 80°C for 30 min. Once dry, the nylon membranes were processed for immunological detection by first washing them in buffer 1 [100mM Tris-HCl, 150mM NaCl, pH 7.5] at room temperature for 1 min. The membranes were next blocked for 30 min in buffer 2 (1% (w/v) casein in buffer 1) at a volume of 1 ml/cm^2. The membranes were then placed in antibody-conjugate solution [(1:5,000) dilution of anti-digoxigenin alkaline phosphatase in buffer 2] for 30 min at room temperature. The samples membranes were then washed twice for 15 min in buffer 1 to remove excess antibody conjugate, followed by equilibration of the membranes for 2 min in buffer 3 [100mM Tris-HCl, 100mM NaCl, 50mM MgCl2, pH 9.5]. Forty-five microliters of the NBT solution [1.25 ml of 75 mg/ml nitroblue tetrazolium salt in dimethylformamide, 70% (v/v)] and 35 ul of the BCIP solution [50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate, toluidinium salt in dimethylformamide] were added to 20 ml of buffer 3. Colorimetric detection of the dot blots using nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt, (NBT-BCIP) lasted for 15-30 minutes until the last pBR328 standard began to appear. At this time the color reaction was stopped using immunological buffer #4 [10mM Tris-HCl, 1mM EDTA, pH 8.0]. Newly prepared probe was quantitated by comparing dot intensities. These dot intensities were compared in an attempt to match the weakest new probe dilution

to a corresponding control pBR328 dot of equal intensity. By comparing these two rows of dots the concentration in ng/ul of the newly labeled probe could be calculated.

Prelabeled probes

Also two commercially available prelabeled probes were chosen. The probes YNH24 and TBQ7 were purchased from Promega and were part of their Gene Print Light™ chemi-luminescent system. Both probes came prelabeled with alkaline phosphatase directly linked to each of them. A synopsis of each probe is listed below.

The probe YNH24 or (D2S44) sequence was originally isolated as a unique cosmid from a total human cosmid library screened with HBV-2 oligonucleotide (GGAGTTGGGGGAGGAG). YNH24 corresponds to chromosomal location D2S44 (17). Quality control on the probe demonstrates that in Southern blot hybridization with a human K562 DNA *Hae* III digest, allele sizes of 2909 bp and 1794 bp are detected. With the Gene Print Light™ system allele sizes may vary 50-100 bp in user's hands.

The probe TBQ7 or (D10S28) sequence is derived from a random cosmid isolated from a library of somatic hybrid cell line 762-8A DNA; this cell line included human chro-mosome 10 and Y(5). In the Gene Print Light™ system, quality control on the probe dem-onstrates that in Southern blot hybridization with a human K562 DNA *Hae* III digest, allele sizes of 1740 bp and 1178 bp are detected.

Hybridization Genius™

Prior to hybridization the membranes were prehybridized in 20 ml/100 cm^2 of hy-bridization solution for 1 hr at 68°C. Hybridization of the membrane bound DNA occurred for 16-20 hr overnight at 68°C in a rotisserie oven. The probe concentration was set at 10 ng/ml in prewarmed hybridization solution [7.0 %SDS, 50mM sodium phosphate (pH 6.8), 2% casein block, and 0.1% N-laurylsarcosine]. Prior to addition, the probe was denatured in a boiling water bath and immediately placed on ice. The hybridization solution volume

was set at 2.5 ml/100 cm^2 of membrane. After hybridization the membranes were washed twice for 15 min at room temperature in low stringency wash #1 [2X SSC, 0.1% SDS (w/v)] at a volume of 50 ml/100 cm^2 of membrane. The final high stringency wash #2 [0.5 X SSC, 0.1% SDS (w/v)] was performed at 65°C and consisted of two 30 min washes at a volume of 50 ml/100 cm^2. After stringency washes the filters were air dried.

Hybridization Gene Print Light™

Prior to hybridization the membranes were blocked for 15 min in freshly prepared blocking solution [2 mg/ml casein in 0.5X SSC] at 55°C. The probe (TBQ7 or YNH24) was added to the prewarmed hybridization solution [5X SSC, 0.2% SDS, 0.1% Ficoll™ , 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.02% sodium azide] at a concentration of 4 ul/ml of hybridization solution. The hybridization solution was used at 50 ul/cm^2 membrane. The Gene Print Light™ ladder was added at a concentration of 2 ul /ml of ladder probe to hybridization solution. The GIBCO BRL ladder probe was added at a concentration of 0.5 ul/ml of hybridization solution. Hybridization occurred for 45 min at 55°C in a rotating hybridization oven. After the first 10 min of hybridization the bottles were burped to prevent pressure build up inside the bottles. Following hybridization the old probe and hybridization solution were poured off and saved, and the bottle was filled with 0.22 um filtered high stringency wash buffer #1 [0.5X SSC, 1% SDS] at a volume of 1.5 ml/cm^2 of membrane. The membrane(s) were then washed for 10 min at 55°C in the hybridization oven. This solution was then decanted, and a second identical high stringency wash of buffer #1 for 10 min at 55°C was performed. The second wash solution was decanted and a third low stringency wash with 0.22 um filtered buffer #2 [0.5X SSC] was added. Buffer #2 was added at a volume of 1.5 ml/cm^2 and washed in a rotating hybridization oven for 10 min at room temperature. Finally the wash buffer #2 was decanted and the nylon membranes were washed in 0.22 um filtered 1X equilibration buffer [(20X)1M carbonate buffer (pH 9.35),

20mM MgCl2] at a volume of 0.6 ml/cm^2. Note: The 1X equilibration buffer comes as a 20X concentrate and changes to a pH of 9.6 on dilution.

Immunological blocking Genius™

This procedure was the same as noted in the probe quantitation protocol of materials and methods. Note: Buffers 1 and 2 and 3 were the same as listed in the probe quantitation section of materials and methods with slight modifications. Buffer 2 had the casein concentration increased to 2% and was spiked with 50 ug/ml of denatured salmon testes DNA. Blocking time with buffer 2 was increased from 30 min to 3 hrs. Incubation with the anti-digoxigenin alkaline phosphatase conjugate in buffer 2, washing twice with buffer 1, and equilibrating in buffer 3 were exactly the same. The membranes were now ready for Lumi-Phos™ 530 detection or NBT-BCIP detection.

NBT-BCIP immunological detection Genius™

For each 100 cm^2 of membrane colorimetric detection with NBT-BCIP after hybridization involved placing 45 ul of NBT solution [1.25 ml of 75 mg/ml nitroblue tetrazolium salt in dimethylformamide, 70% (v/v)] and 35 ul BCIP solution [50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate, toluidinium salt in dimethylformamide] into 10 ml of immunological buffer #3 [100mM Tris-HCl, (pH 9.5) 100mM NaCl, 50mM MgCl2]. Exposure occured at room temperature in a dark area for 1-24 hrs until the desire band intensity was obtained. The color reaction was stopped with immunological buffer #4 [10mM Tris-HCl (pH 8.0), 1mM EDTA] by placing membranes in this solution for 10 min..

Lumi-Phos™ 530 detection

Incubation times with Lumi-Phos™ 530 were extended to overnight using XAR X-ray film (21). At the beginning of this project, Lumi-Phos™ 530 was initially sprayed on the membranes with an aerosol sprayer and placed between two acetate sheets. Once the Promega system was incorporated however, the Lumi-Phos™ 530 application changed.

One and a half ml of Lumi-Phos™ 530 was pipetted down on an acetate sheet folder for each 11 x 15 cm membrane. The membrane was dipped in at one end allowing the solution to run to the other end. The top acetate sheet was placed on top and a glass rod was rolled over to press out the excess solution. To prevent drying out of the membrane, the edges of the acetate sheet were sealed with cellophane tape.

X-ray film development

Visualization was by use of Kodak X-OMAT AR film. Kodak GBX developer and fixer was used according to manufacturers directions for film processing. Tray development was performed using 500 ml each of developer, stop bath and fixer. A development temperature of 68°F with a 5.0 min development time was followed by a 30 sec stop bath in room temperature 2.5 % acetic acid. A 3.0 min fixation at room temperature was followed by a 10 min wash in running tap water. Processed films were rinsed once in distilled water and air dried. Chemistry was prepared fresh daily processing 1-4 films.

Stripping and reprobing

After X-ray film exposure, the membranes were rinsed in sterile distilled water for 10 min to remove the Lumi-Phos™ 530. The membranes were then incubated in 0.1% SDS at 65°C for 45 min in a rotating hybridization oven to strip off the old probe. The membrane was then stored dry for later use.

RESULTS AND DISCUSSION

Extraction

Difficulties encountered with the high salt extraction procedure could not be resolved. Yields were low and not reproducible. Part of this was due to inexperience with the procedure; however, the procedure did not appear to have the same recovery rate as expected (12). The organic extraction did work very well and showed great reproducibility. Therefore the organic extraction procedure was used in the second and third round of DNA sample extractions.

FBI Yield gel and digestion gel

Figure 1 shows a typical DNA quantitation gel. A double origin is used so up to 24 samples can be applied per gel. An organic phenol/chloroform extraction was performed followed by ethanol precipitation. Samples were then resuspended in Tris EDTA(TE) buffer for application to the gel.

Figure 2 shows a typical *Hae* III restriction gel. A double origin is also used here so 24 samples can be applied per gel. All human DNA samples were restricted overnight at 37°C with a 5-8 fold excess of *Hae* III enzyme. Samples were ethanol precipitated and resuspended in TE buffer.

Analytical gel

Some of the initial analytical gels were run on a 20 x 20 cm Pharmacia horizontal submarine tank, and on the 7.0 x 9.2 cm Fotodyne mini-gel tanks. The reason for their use, especially the Fotodyne mini-gel tanks, was to work out problems with the procedure. A smaller format gel consumed fewer reagents. With the use of the Promega Gene Print Light™ system, the newer 11 x 16 cm GIBCO BRL analytical tanks were used.

Monitoring the levelness of the gels and electrophoresis tanks was important. A difference in the electrophoretic mobility would be noticed from one end of the gel to the

other. Gels were poured on a leveling table, and all electrophoresis tanks were placed on a level counter top during electrophoresis. The level of buffer running over the top of the gels during electrophoresis was closely monitored so gel to gel electrophoresis did not vary significantly. If one gel had significantly more buffer running over the top of it during electrophoresis, the DNA fragments would travel a shorter distance in this gel since more current would be running through the buffer, and less current through the gel.

Another problem noted during the analytical gel preparations was not letting the gels solidify hard enough before removing the combs. On two occasions removal of the combs too early resulted in the wells closing up partially on the bottom preventing the proper filling of all the sample into the wells. Once the gels were poured, they had to sit at least 15 min before moving them or attempting to remove the combs. This was especially true during the summer months when the laboratory was warmer.

Since the digoxigenin system was eliminated, new ladders were chosen. After examination the GIBCO BRL Photo Probe™ direct link ladder was chosen. This was a 30-band ladder labeled with alkaline phosphatase (A-P) and was compatible with the Promega Gene Print Light™ system. The high band number in this ladder made it an excellent analytical ladder for case work. One problem with this combination however, was that Promega called for a 55°C hybridization for their probes and BRL called for 50°C hybridization for their ladder (1, 20). A compromise of 52.5°C was chosen.

Because of the hybridization temperature difference and a later change back to the Gene Print Light™ hybridization solution another ladder was chosen. The Promega Gene Print Light™ Equiladder which contained 29 bands from 485 bp to 25.632 kb was compared to the GIBCO BRL Photo Probe™ ladder which contained 30 bands from 526 bp to 22.6 kb. Both ladders were direct labeled with alkaline phosphatase which made them much easier and faster to use.

Another time saving decision was to use *Hae* III digested human K562 DNA as samples to experiment with sensitivities on the analytical gels. This was done when the Promega system was begun. This gave consistency of sample, and a known concentration to work with.

Southern transfer

Southern blots in the Promega system used a high salt 10X SSC transfer from 6 hrs to overnight, using only 4 blotting pads instead of sponges. The manufacturer further reported that Southern blots could be run in 1 hr with satisfactory results. Southern blots in this project were run from 2-6 hrs with no observable loss in sensitivity. A Southern blot time of 5.0 hrs with sponges was chosen based on ease of scheduling.

The choice of MSI Magnagraph membranes was made because Boehringer Mannheim was using them with their system. Examination of these membranes showed that the MSI Magnagraph was one of the best suited membranes for chemiluminescence since it was a slightly positively charged to neutral membrane. This made it ideal for chemiluminescence and a neutral high salt Southern transfer. An alkaline blot with a highly positively charged membrane binds the DNA strongly to the membrane with an 80°C heat bake for 30 min (1). However, for neutral membranes one must U-V fix the DNA and heat bake to get the DNA to fix well when using the high salt transfer. The changing of these variables could significantly affect the sensitivity and background of the systems. For this reason the Biodyne B membranes (Pall BioSupport) were not used as in the FBI protocol. The Biodyne B membranes were highly positively charged and were designed for an alkaline transfer.

New information in the latest Current Protocols in Molecular Biology suggested that optimizing DNA fixation to the membrane through U-V exposure and/or heat baking would make a considerable difference on signal strength when rehybridizing membranes. This was especially true when using chemiluminescence detection in conjunction with a high salt

Southern transfer and a neutral membrane. For this reason the dot blot fixation membranes in Figures 9, 10, and 11 were run. The results as stated earlier in the material and methods show that the membrane should be dried before U-V fixation, and that a combination of U-V fixation with heat baking at 80°C improved signal strength. This work applied only to the MSI Magnagraph nylon membrane, and may vary if differing conditions or a different membrane were used.

Fixation

Another problem encountered with the analytical gel work was lack of sensitivity. A lack of DNA binding, apparently due to poor fixation of DNA to the nylon membranes was noted. This was cited as a problem in the literature (1). The type of fixation for binding DNA to the Southern blotted membrane affected sensitivity. U-V fixing membranes and /or heat baking membranes in a vacuum oven significantly affected the binding of DNA. A series of experiments was performed to determine whether U-V fixing or heat baking would enhance DNA binding to a non-charged nylon membrane. Current Protocols in Molecular Biology (1) suggested empirically calibrating the U-V source to determine the optimum time of U-V exposure. If the DNA was exposed too little, the DNA would not bind properly to the membrane, and if the DNA was exposed too long, DNA would be damaged. A dot blot procedure using the ACES™ Human DNA quantitation system was used to determine the optimum U-V exposure time. A set of dot blots using 3.2 ng of human DNA was spotted eight times to vary U-V exposure time from 30 sec to 5.0 min. Also concentrations of human DNA ranging from 0.16 ng to 3.2 ng were spotted on a nylon membrane and all spots were exposed for 1 min. Samples were hybridized according to manufacturers instructions and visualized with Lumi-Phos™ 530 and Kodak XAR X-ray film. A 1.0 hr exposure was performed after an overnight ramp time of 16 hrs.

A comparison of time factors for detection began after the transfer of DNA to the

MSI Magnagraph nylon membrane. Ultraviolet exposures were made with a Fisher model FBTIV-88 viewing box. Exposures were made at: 30, 45, sec, 1, 2, 3, 4, and 5 min using 3.2 ng per well at 254 nm. Rows were air dried or heat baked at 80°C for 30 min. The row that was both heat baked and U-V fixed showed considerably greater dot intensity on the film. This was especially true for the rehybridized dot blots. Also the U-V exposure at 5 min showed a decrease in dot intensity indicating that the DNA had been over exposed. There was actually little difference in the comparison of the air dried vs. heat baked membranes after just one probing. From the results it appeared that an exposure time of at least 1.0 min was needed for fixation. The other variable of using different concentrations of DNA on a membrane ranging from 0.16 ng-3.2 ng and exposing the membranes to U-V fixation in a damp state versus a dry state for 1.0 min demonstrated both on an initial hybridization and after being stripped and rehybridized that the dry U-V fixation retained better signal on the films. For further work a 30 min heat baking at 80°C followed by U-V fixation at 254 nm for 1.5 min was chosen. This seemed to retain signal better than either one by itself (1). See figures 9, 10, and 11 comparing air dry vs. heat bake before U-V fixation at 254 nm and comparison of damp vs. dry U-V exposure.

The Fisher FBTIV-88 U-V box was used to empirically determine the optimum time for U-V exposures; however, most U-V fixation problems were solved with the purchase of the Fisher FBUVXL 1000 Crosslinker. The Fisher FBUVXL 1000 U-V Crosslinker determined the correct exposure time by itself. An auto setting was attained since the instrument had a sensor in the bottom of the oven to monitor the actual exposure, even if the bulbs lost power over time.

The use of the FBUVXL1000 U-V box in combination with heat baking the nylon membranes at 80°C for 30 min showed an increase in signal strength, during successive stripping and rehybridization. This was shown in the membranes developed on 3-19-93.

Probe labeling

Probe labeling was a new procedure that had never been performed by the DCI laboratory. Since the probe pH30 had proven very sensitive, it was selected for the project. Random primer extension was the only method available for the Genius™ kit at the time of the project. Probe labeling was performed according to the Boehringer Mannheim protocol. An overnight or extended random primer extension time was run overnight to produce a higher concentration of probe. (See B-M insert). Precipitation of the probe was performed according to manufacturer instructions, using glycogen and NaCl . Note: NaCl replaced the lithium chloride (LiCl) for probe precipitation and clean up. Because of inexperience in probe labeling a *Hind* III digested lambda DNA probe was produced to work out background problems in the hybridization and immunological detection steps. Concentration of this lambda probe was set at 5-10 ng/ml for hybridization when using chemiluminescence.

Initial problems holding the probe down for denaturing just before labeling was resolved by using a screw top 1.5 ml microcentrifuge tube from Sarstadt that contained an o-ring rather than flip top. These tubes with probe were placed in a home made tube holder to keep the tube immersed in a boiling water bath for 10 minutes to denature the double stranded DNA. The homemade apparatus kept the tube upright while staying completely immersed in the boiling water bath.

Probe quantitation

For a 10 ml hybridization solution, 50-250 ng of probe needed to be used. With a stepped up 50 ul probe labeling reaction , 4 ul or 120 ng of *Hind* III cut DNA were labeled using the *Hind* III cut lambda DNA, while 100 ng of pH30 probe were set up in the standard 20 ul probe labeling reaction mixture. Approximately 50 ng of DNA was recovered showing a 42% yield for the *Hind* III cut lambda DNA probe. The pH30 probe labeling was

approximately 250 ng the first time, and only 2.5 ng the third and fourth times. No further probe labelings were performed since the pH30 probe was consumed. Figure 3 shows a quantitation dot blot membrane. The probe pH30 is being quantitated according to the Boehringer Mannheim Genius™ protocol. Probe labeling, precipitation and resuspension were also performed according to manufacturer's instructions. Figure 4 shows another quantitation dot blot membrane. The lambda DNA probe was quantitated according to the Boehringer Mannheim Genius™ protocol. Probe labeling, precipitation, and resuspension were also performed according to the manufacturer's instructions. Digoxigenin labeled lambda probe was produced with no problem. Numerous preparations and quantitations of lambda probe were performed to work out problems with probe labeling and background spots.

Since only a small amount of digoxigenin labeled pH30 probe was prepared, membrane sizes for hybridization had to be reduced. Only small membranes were probed with pH30 since at a concentration of 10 ng/ml usually 5-10 ml of probe were needed to hybridize a membrane. The protocol recommended 2.5 ml of probe per 100 cm^2 of membrane. The probe concentration was decided empirically after numerous hybridizations. Four batches of probe were made with limited success. Probe yields from labeling were always very poor. Frequent conversations with the B-M technical department suggested that the probe should label and that practice should be done labeling pBR328 as a probe. This was done with no problem. GIBCO BRL, the vendor for the pH30 probe suggested that the probe was not designed for digoxigenin labeling with a dig.-UTP, (digoxigenin-UTP), since it was a G-C rich probe (15). Therefore it would never label well. My understanding of the digoxigenin-UTP label in the probe labeling strategy is that by using a uracil for incorporation, this random primer labeling kit can be used for both DNA and RNA systems. However since this probe contained only one adenine, and five thymines, it would never theo-

retically work well unless the labeling digoxigenin was attached to a different base such as a guanine or cytosine. GIBCO BRL did suggest lowering labeling temperature from 37°C to 25°C or using decamers instead of hexamers for the incorporation. This was due to the melting point (Tm) of the probe. This was tried with little success. Boehringer Mannheim also suggested reextracting and reprecipitating the probe. All these attempts were futile.

Only one batch of legible films were visualized with high backgrounds and weak results. See the mini-gel results in figure 5 of a pH30 hybridization of membranes A, B and C from 5-11-92 using a 1.0 hour incubation with Lumi-Phos™ 530 after a 30 min preheat at 37°C. The films in A, B and C are from 3 separate mini-analytical gels. Samples are human DNA digests from 2-16 ug and the digoxigenin labeled Boehringer-Mannheim Molecular Weight Marker III, (B-M MWM III). Membranes from Figure 6 show the same membranes as Figure 5, but with a 4.0 hr Lumi-Phos™ 530 exposure. Note the increased signal strength but also the increased background. This background problem suggested a series of experiments on optimizing membrane blocking.

Prelabeled probes

After depletion of pH30 probe and difficulties with background, a new probing procedure was examined. This procedure used a direct link system rather than an antigen antibody conjugate technique. Promega Gene Print Light™ system used direct link oligonucleotide probes (20). The Promega probes had the alkaline phosphatase enzyme bound directly to the probe. Advantages to this system were: no denaturing since it was an oligonucleotide probe, no secondary antibody since it was a direct link probe, reduced detection time and lower cost. The oligonucleotide direct link probes YNH24 and TBQ7 were examined. Also it should be noted that GIBCO BRL has now marketed the probe pH30 (D4S139) as a direct link alkaline phosphatase conjugate probe which was not available during the start of this project.

Hybridization Genius™

Hybridization of probe to membrane bound DNA initially was a big problem. Zip lock bags containing probe and membranes were placed in a 60°C water bath sitting on a shaker. This did not appear to be a safe apparatus so an alternate homemade hybridization oven was created. This consisted of taking a Precision gravity convection incubator and attaching a grill rotisserie through the center of it . This was accomplished by drilling holes through the sides and attaching a grill motor to the outside of the oven and running the rotisserie shaft through the center of the oven. The next problem with this was the attachment of a hybridization bottle to the rotisserie. Large rubber bands were used by wrapping them around the rotisserie shaft and hybridization bottles. A series of hybridization bottles were designed with limited success.

Four different hybridization bottles were created. All hybridization bottles were attached to the rotisserie by two heavy rubber bands. First, a 50 ml conical centrifuge tube was used. This was a polypropylene tube that appeared to be air tight, but tended to leak after reaching 60°C over extended times. The next bottle consisted of a 250 ml wide mouthed polypropylene bottle with an o-ring placed in the lid to help seal the bottle. This did not seal well either. A third 100 ml glass baby bottle with a cut out rubber O-ring was attempted with little success. Finally a hybridization bottle was purchased from Midwest Scientific. This Hybaid brand bottle was made of borosilicate glass and was 15 cm long x 3.5 cm in diameter, and was treated with repel silane to prevent DNA from adhering to the inside walls of the bottle. The bottle was also air tight with a heavy duty gasket in the lid. The Hybaid bottle worked well for hybridization.

To keep all these bottles from leaking during hybridization at high temperature, the bottles and their contents had to be prewarmed to prevent pressure from building up. Also the bottles were burped after 10 min to equilibrate pressures. Another problem with the

homemade hybridization oven was that the rubber bands on the rotisserie broke during the overnight hybridization, leaving the hybridization bottles on the bottom of the oven and preventing probe circulation around the membrane. Also it was difficult to maintain the high temperature for hybridization since there was no fan to maintain a uniform temperature. Eventually an actual hybridization oven with a built in rotisserie was purchased from Hybaid via Midwest Scientific including 4 full sized glass hybridization bottles 30 cm long x 3.5 cm in diameter.

Problems also arose with air bubbles in the new hybridization oven. Attempts to eliminate them included placing a glass rod in the bottle as a roller to help push out the air bubbles between the membranes and the side of the glass hybridization bottle. If the air bubbles remained, no probe would hybridize to that spot on the membrane. Rolling in the correct direction also helped with the problem. If bottles were put in the wrong way, the membrane would roll in upon itself. Also the placement of mesh between the membranes helped eliminate this problem.

Hybridization Gene Print Light™

Hybridization and hybridization blocking solutions were chosen according to FBI protocol and Current Protocols in Molecular Biology based on cost and ease of use (1, 7). Blocking was done using the FBI hybridization solution for 15 min and then adding the probe at 52.5°C. Blocking and hybridization times were used according to the Promega protocol using a Hybaid revolving hybridization oven. All solutions and bottles in these steps were prewarmed to temperature. All wash times, temperatures and solutions were kept constant. This choice was made in an attempt to follow the FBI protocol as much as possible. Due to the problems with background it was decided to drop the FBI hybridization solution, use the Promega hybridization solution (0.2% casein), and prepare the blocking solution according to manufacturer's instructions. This solution could be saved for

more than 2 days by adding sodium azide at 0.02%. Another advantage to this was that an 87% recovery rate was made using the Promega hybridization solution rather than the FBI hybridization solution which usually only recovered 50%. At this point, the hybridization temperature was set back to 55°C. The Gene Print Light™ ladder also became the ladder of choice.

While using the FBI hybridization solution, a decrease in sensitivity and increase in background was noted with rehybridizing membranes with other probes using the Promega Gene Print Light™ system. Probe was saved and respiked with fresh probe to get back to the recommended concentration. This needed to be done since the recovery rate of the FBI hybridization solution after probing was very poor. After saving batches of probe, one labeling was tried using just old labeled probe. The sensitivity was unacceptable. Both manufacturers said a drop off in sensitivity would be four fold for six uses in 2 weeks. At this time it was not known if this was due to poor U-V fixing, poor labeling, or poor rehybridizing. Another factor may have been with the FBI hybridization solution which was not recommended for use with the Promega system.

Immunological blocking

The background problems with the Genius™ system were a known factor but it was not known how significant these problems were. A series of different approaches were used. Increasing the concentration of the prehybridization blocking reagent, increasing the concentration of the immunological blocking reagent and extending these blocking times were all done according to the manufacturer's updated instructions. Also the addition of 50 mg/ml of denatured salmon testis DNA to the immunological blocking reagent was done to act as a non-human DNA source for blocking. Note: This was a substitute for the recommended herring sperm DNA. The use of Uni-Block from AGTC for a prehybridization and immunological blocking reagent was also attempted with good success. The reason for this

switch over during the immunological blocking steps was that the casein based Genius™ blocking reagent needed to be made fresh daily which took a minimum of 1.5 hrs for preparation. The Uni-Block came as a ready to use concentrate which saved time. This Uni-Block could not be used for hybridization since it could only be heated to 45°C. The advantage of Uni-Block was that it had a shelf life of 6 months at 4°C.

A series of varying concentrations and incubation temperatures for varying times were performed comparing the Uni-Block versus the casein blocking reagent. Optimal times, temperatures, and concentrations of the blocking reagents were obtained with the lambda DNA probings.

With the Boehringer Mannheim Genius™ system membrane blocking must be performed at two stages. First, the membrane was blocked just prior to hybridization. Second, the membrane was blocked after hybridization and before immunological detection with the anti-digoxigenin alkaline phosphatase conjugate. Prehybridization and immunological blocking reagents in the Genius™ kit used casein. Uni-Block, from Analytical Genetic Testing Center (AGTC) was substituted for casein in an attempt to reduce background problems. Figures 7 and 8 show a comparison of the two reagents. The Figure 7 membranes used casein for both the prehybridization and immunological blocking while the Figure 8 membranes used Uni-Block for both the prehybridization and immunological blocking. No observable difference was noted between the two blocking reagents. The Uni-block worked, but was not any better. The gels in Figures 7 and 8 contained identical samples of lambda DNA and Boehringer Mannheim's Molecular Weight Marker III ladder, (MWM III).

Lumi-Phos™ 530

The Lumi-Phos™ light emission time course showed that light production increased for the first 7-8 hrs, leveled off for the next 3-4 hrs, and slowly decreased over the next 96 hrs. For this reason it was important to establish a consistent time frame for incubation

since the light output varied dramatically over the first 48 hrs.

Mirrors

Another attempt to increase sensitivity was the by use of mirrors. The membrane and X-ray film were sandwiched between mirrors as mentioned in the Boehringer Mannheim updates (3, 4). Initial work with these were not successful because the background levels were too high. Further work with these mirrors using the Promega Gene Print Light™ showed a slight increase in sensitivity; however, the bands became broader, and the background still increased.

The use of mirrors on both sides of the membranes and films did show some increased signal strength, but background also increased. One needs to assess the point at which background overtakes the samples (3, 4). Figure 13 showed this with a 7 hr exposure. (A longer 20 hr exposure needed to be compared.) The films show probing of 2 membranes with YNH24 and samples of human K562 digests with both the Promega Gene Print™ Light ladder and the GIBCO BRL ladder. The 7 hr Lumi-Phos™ 530 exposure was made immediately after the 20 hr exposure. Membrane 13A had mirrors on either side to enhance the bands, and membrane 13B did not. Both of these membranes were prepared using fresh probe in new hybridization solution. These gels were prepared 3-10-93, and developed 3-19-93.

Using mirrors may help with increasing the detection limits of chemiluminescent systems, but this work did not show a significant effect. The use of a front reflective surface mirror rather than a back reflective mirror should enhance results. The cost of these mirrors however, may be prohibitive.

X-ray film development

During the summer, development temperature was set at 74°F. Times were set at 3.5 min development, 30 sec stop, 3.0 min fixation, and 10 min wash in tap water. The reason

for the temperature change in development times was due to the increased water temperature of incoming water during the summer months. No water cooler than 74°F was available from the faucets. All washes fixes and developments were performed in the photographic section of the DCI laboratory since the sinks, trays, darkrooms and water regulators were available in that area. Problems were noted with developing more than 2 films at a time. Scratches were made in the films during the developer stage, and fingerprints showed up on the films which indicated that emulsion was being pushed off the films with the finger tips. Latex gloves did not work either because dexterity to manipulate films was lost with them on. Possibly not processing so many sheets at a time would have helped.

Examination of nearly all developed X-ray films revealed numerous random spots where the membranes were exposed on them. The spots were thought to be: 1) bacterial alkaline phosphatase activity reacting with the Lumi-Phos™ 530, 2) precipitated probe settling out on the membrane during hybridization, or 3) Lumi-Phos™ 530 deposits with impurities. To eliminate spots, all SSC wash and rinse buffers were autoclaved. Also all low stringency, high stringency and equilibration wash solutions were vacuum filtered with a 0.22 um nylon filter unit from Micron Separations Inc., (MSI). A mini-experiment using different hybridization, wash and equilibration solutions in differing combinations indicated that the final wash solution was causing part of the problem. The GIBCO BRL Aces™ 1X final wash solution was substituted for the Genius™ immunological buffer number three. After these findings, all handling and washing of the hybridized nylon membranes was done in a sterile environment. In retrospect, initial work with the Genius™ kit and the background problems may have been associated to the final wash buffer not being sterile; however, no sterilizer or filtering devices were available at that time. This knowledge could have resolved some of the early problems with the Genius™ system.

After consultation with other DNA scientists in April 1993, many felt the back-

ground problem was the result of precipitated probe settling out on the membranes. Suggestions included centrifugation of the probe before use according to the Promega protocol, or the addition of denatured herring sperm DNA at immunological blocking time (1, 7).

Even though the nylon membranes were much more durable than nitrocellulose membranes, care needed to be taken when handling them. Any strong pressure on the membranes would leave marks which would show up on the X-ray films as dark spots. This could include forceps marks, finger tip marks, or kinks in the membranes.

Probe stripping and rehybridization

The rehybridizing of membranes from 2-08-93 with probe YNH24 showed much less background and better visibility of K562 sample dilutions. The Gene Print™ ladders from Promega did show a drop off in sensitivity. This may be due to either rehybridization or poor binding to the membranes by fixation methods. Detection at 100 ng were barely visible however, 500 ng were easily read after a 20 hr exposure with Lumi-Phos™ 530 on x-ray film. Another set of membranes developed for 23 hrs on 3-18-93 from two 3-10-93 gels is a rehybridization of two membranes using both TBQ7 and YNH24. See figure 14. Both probes worked quite well. These membranes then had the Lumi-Phos™ 530 washed off with distilled water, and then were colorimetrically developed using NBT-BCIP for 24 hrs. See figure 15. The background is much less with this system, but the sensitivity is down considerably. Detection is down to approximately 500 ng with YNH24, and approximately 1.0 ug using TBQ7.

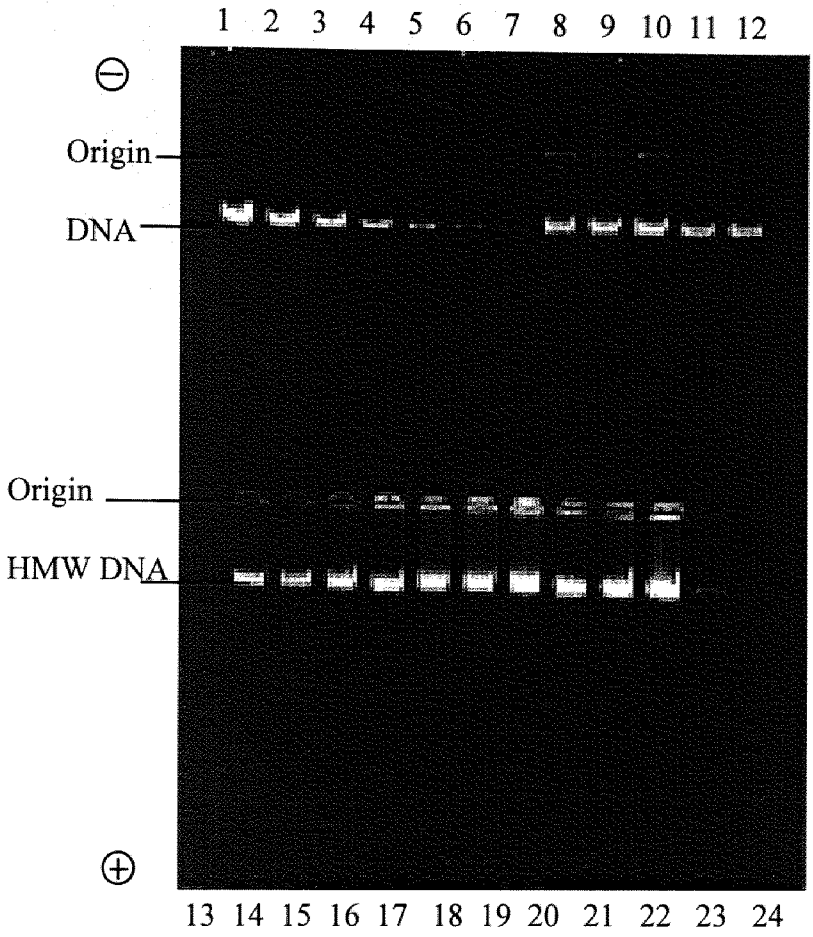

Fig. 1 Double origin quantitation gel from 9-01-92: Row 1 contains 12 samples. From left to right are BRL lambda DNA quantitation standards from 500 ng-15 ng in lanes 1-6. Lane 7 contains 120 ng of *Hind* III digested lambda phage DNA, and lanes 8-12 contain human stain extracts from approximately 63-500 ng/sample. Row 2 in center of gel contains 12-samples of human stain extracts from approximately 63-500 ng/sample. Electrophoresis was at 200 volts for 15 min. Gel was 1% agarose in 1X TAE buffer with 0.05 ug/ml of ethidium bromide. Tank buffer was the same as gel buffer. Photograph was at f-8 for 1/2 sec using Polaroid™ 667 high speed black and white film with a red/orange DNA photographic filter. Lighting was with an ultraviolet light source at 302 nm. Note all samples show high molecular weight DNA.

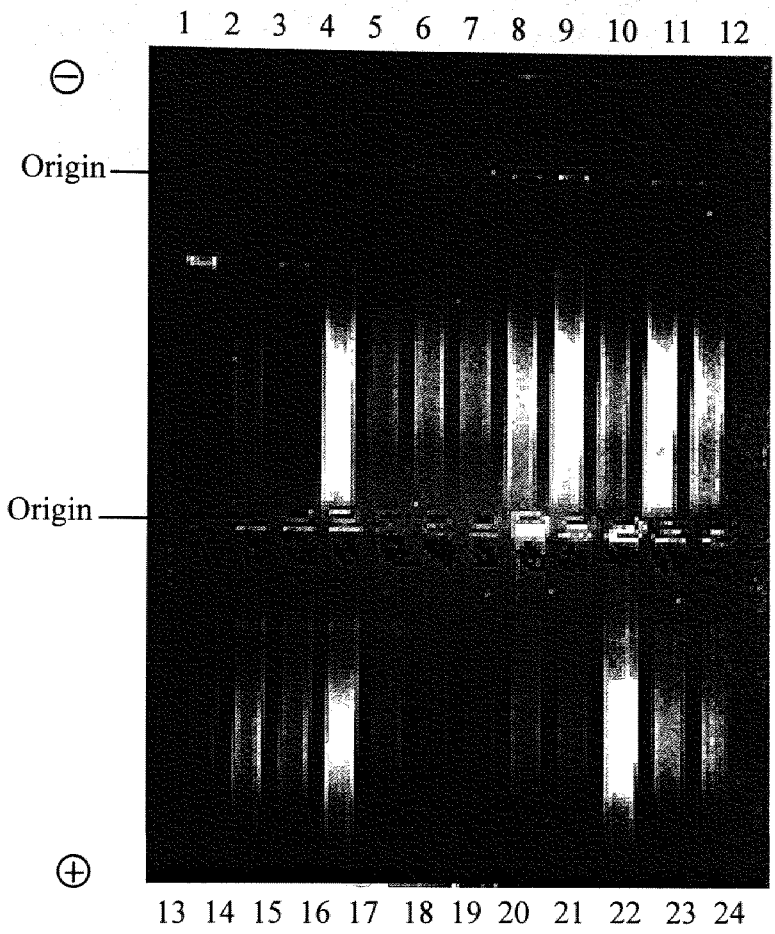

Fig. 2 Double origin *Hae* III restriction gel from 5-07-92: Row 1 contains 12 samples. From left to right are: 1) 200 ng K562 human DNA unrestricted, 2) 200 ng K562 human DNA restricted with *Hae* III., 3) 120 ng of lambda phage DNA restricted with *Hind* III., 4) K562 human DNA restricted with *Hae* III., 5-12) samples of human DNA restricted with *Hae* III. Row 2 in center of gel contains 12 samples of human DNA restricted with *Hae* III. Electrophoresis was at 200 volts for 15 min. Gel was 1% agarose in 1X TAE buffer with 0.05 ug/ml of ethidium bromide. Tank buffer was the same as gel buffer. Photograph was at f-8 for 1/2 second using Polaroid™ 667 high speed black and white film with a red/ orange DNA photographic filter. Lighting was with an ultraviolet light source at 302 nm. Note that all samples digested very well as shown by the uniform streaking in the lanes.

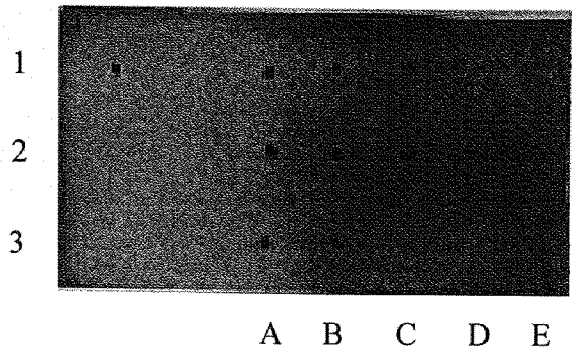

Fig. 3 Quantitation dot blots of digoxigenin labeled pH30 probe from 3-13-92: Row 1 contains 5 serial dilutions of digoxigenin labeled pBR328 standard. Row 2 contains a second preparation of 5 serial dilutions of digoxigenin labeled pBR328 standard. Row 3 contains 5 serial dilutions of freshly prepared pH30 human DNA probe. Concentrations of the pBR328 probe range from 1.0 ng/ul in column A to 0.1 pg/ul in column E. Human pH30 probe was prepared in a 20 ul standard reaction volume. Labeling was run overnight according to Boerhringer Mannheim's instructions. 100 ng of pH30 probe was used in the labeling mixture. The total yield of digoxigenin labeled pH30 probe was approximately 250 ng. The nylon membranes with the spotted DNA probes were heat baked at 80°C for 30 min to fix the DNA. Development was by immunological detection using NBT-BCIP colorimetric detection until the lowest (0.1 pg) pBR328 standard was just visible.

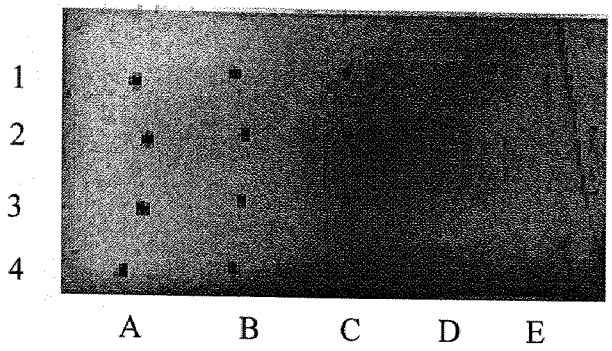

Fig. 4 Quantitation dot blots of digoxigenin labeled lambda probe from 3-13-92: Rows 1 and 2 contain 5 serial dilutions of digoxigenin labeled pBR328 standard. Rows 3 and 4 contain 5 serial dilutions of freshly prepared lambda DNA probe. Concentrations of the pBR328 probe standard range from 1.0 ng/ul in column A to 0.1 pg/ul in column E. Lambda DNA probe in row three was prepared in a 20 ul standard reaction volume. Labeling was run overnight according to manufacturers instructions. 120 ng of lambda phage DNA was used in the labeling mixture. Lambda DNA probe in row four was prepared in a 50 ul stepped up reaction volume. Labeling was run overnight according to manufacturer's instructions. 120 ng of lambda phage DNA was used in the labeling mixture. Total yield from each lambda probe preparation was approximately 50 ng. The nylon membranes with the spotted DNA probes were heat baked at 80° C for 30 min to fix the DNA. Development was by immunological detection using NBT-BCIP colorimetric detection until the lowest (0.1pg) pBR328 standard was just visible.

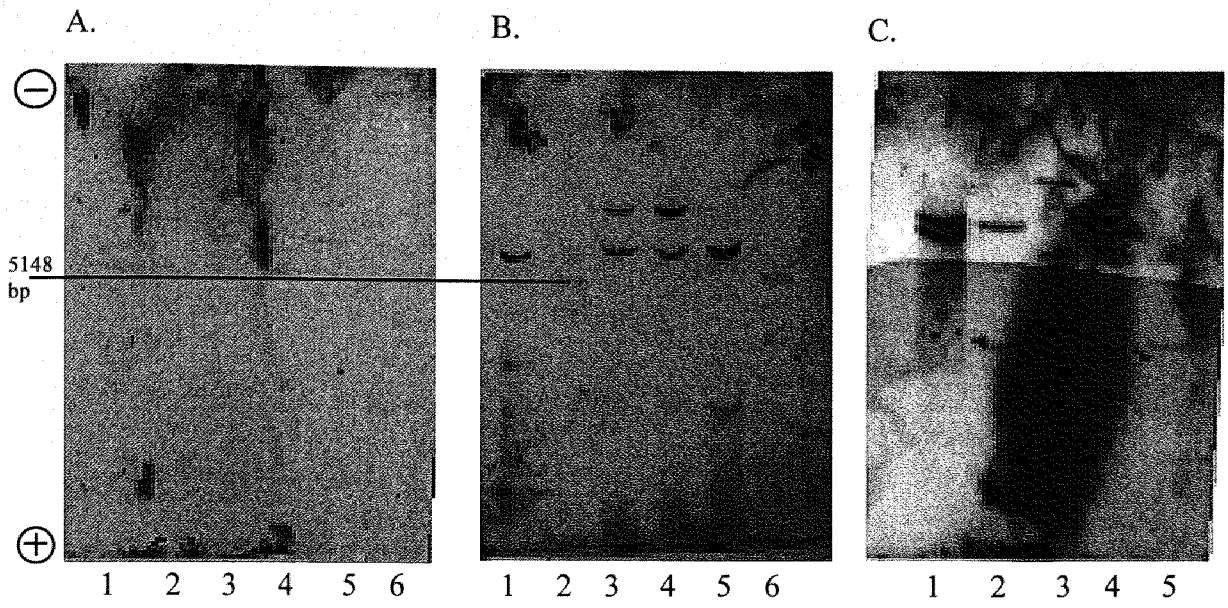

Fig. 5 Chemiluminescent mini-analytical gel films probed with digoxigenin labeled pH30 (1.0 hr Lumi-Phos™ 530 exposure) from 5-11-92: Film 5A contains 6 lanes as follows: 1) 2.0 ug K562 human DNA, 2) 2.5 ng B-M MWM III Ladder, 3) 8.2 ug human DNA, 4) 2.5 ng B-M MWM III Ladder, 5) 4.1 ug human DNA, and 6) 2.0 ug human DNA samples. Film 5B contains 6 lanes as follows: 1) 4.0 ug K562 human DNA, 2) 2.5 ng B-M MWM III Ladder, 3) 4.1 ug human DNA, 4) 8.2 ug human DNA, 5) 16.4 ug - human DNA, and, 6) B-M MWM III Ladder. Film 5C contains 5 lanes as follows: 1) 16.4 ug human DNA, 2) 8.2 ug human DNA, 3) 2.5 ng B-M MWM III Ladder, 4) 4.1 ug human DNA, and 5) 2.0 ug human DNA. All human DNA samples were restricted with *Hae* III. Electrophoresis was at 21 volts for 14 hrs. Gel was 1% agarose in 1X TAE buffer with 0.05 ug/ml of ethidium bromide. Tank buffer was the same as gel buffer. Probe was pH30 at a concentration of 10 ng/ml. Hybridization with probe was for 18 hrs at 68°C. Exposure was with Lumi-Phos™ 530 for 1.0 hr with a half hour prewarm on Kodak XAR X-ray film.

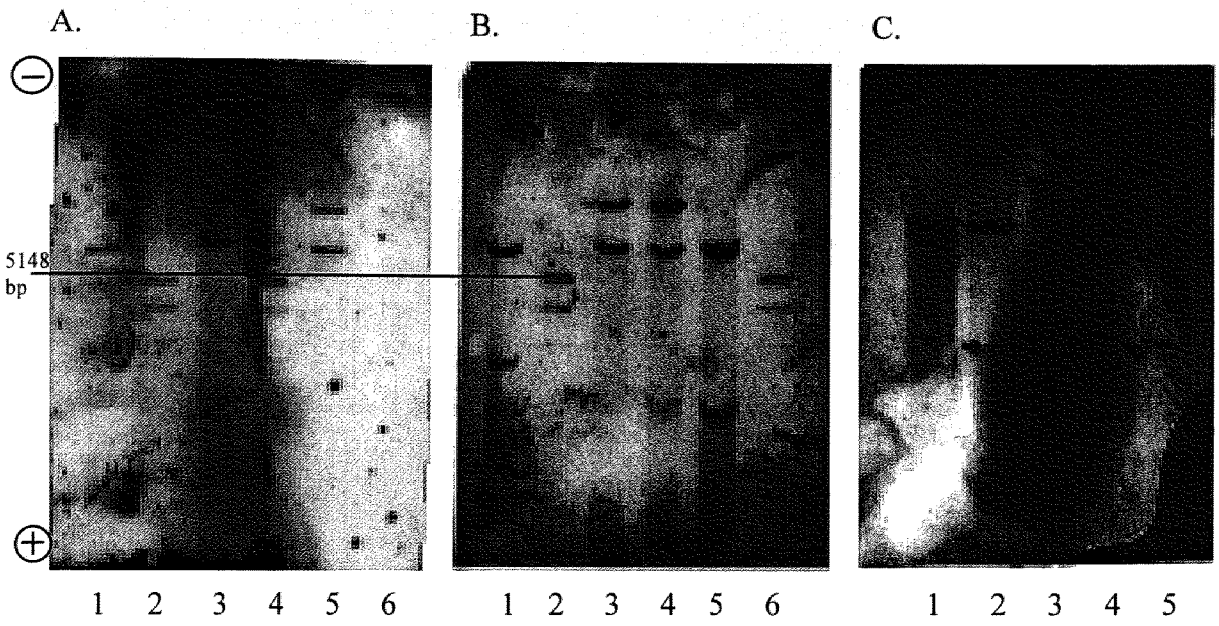

Fig. 6 Chemiluminescent mini-analytical gel films probed with digoxigenin labeled pH30 (4.0 hr Lumi-Phos™ 530 exposure) from 5-11-92: Figure 6A, 6B, and 6C are identical to those in figure 5A, 5B, and 5C. All sample lanes, hybridization parameters, electrophoresis parameters, and probe, are the same. The only difference is that this is a 4.0 hr Lumi-Phos™ 530 exposure.

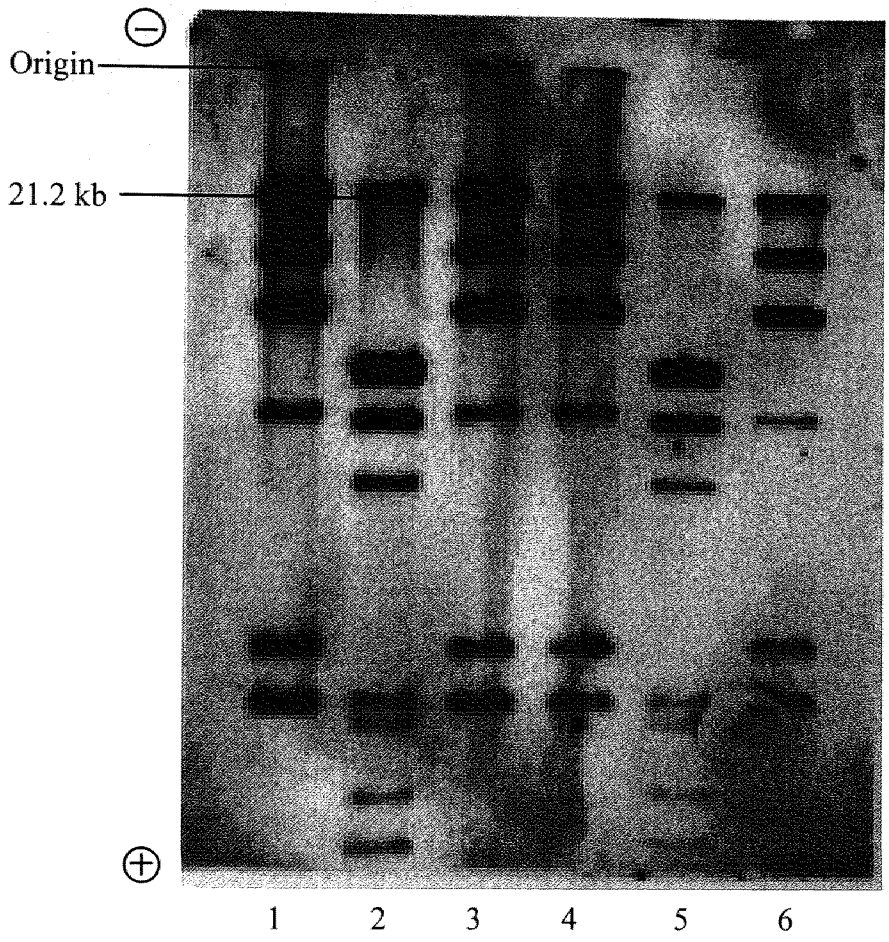

Fig. 7 Chemiluminescent mini-analytical gel film with digoxigenin labeled *Hind* III digested lambda probe and Boehringer Mannheim prehybridization and blocking reagents from 4-02-92: Figure 7 contains 6 lanes as follows: 1) 0.09 ug *Hind* III digested lambda DNA, 2) 0.02 ug B-M MWM III Ladder, 3) 0.045 ug *Hind* III digested lambda DNA, 4) 0.03 ug *Hind* III digested lambda DNA, 5) 0.01 ug B-M MWM III Ladder, and 6) 0.015 ug *Hind* III digested lambda DNA. Electrophoresis was at 22 volts for 12.5 hrs. Gel was 1% agarose in 1X TAE buffer with 0.05 ug/ml of ethidium bromide. Tank buffer was the same as gel buffer. Hybridization was for 18 hrs at 68°C using lambda DNA probe at 5.0 ng/ml. Boehringer Mannheim prehybridization and immunological blocking reagents were used. Exposure was with Lumi-Phos™ 530 for 1.0 hr with Kodak XAR X-ray film.

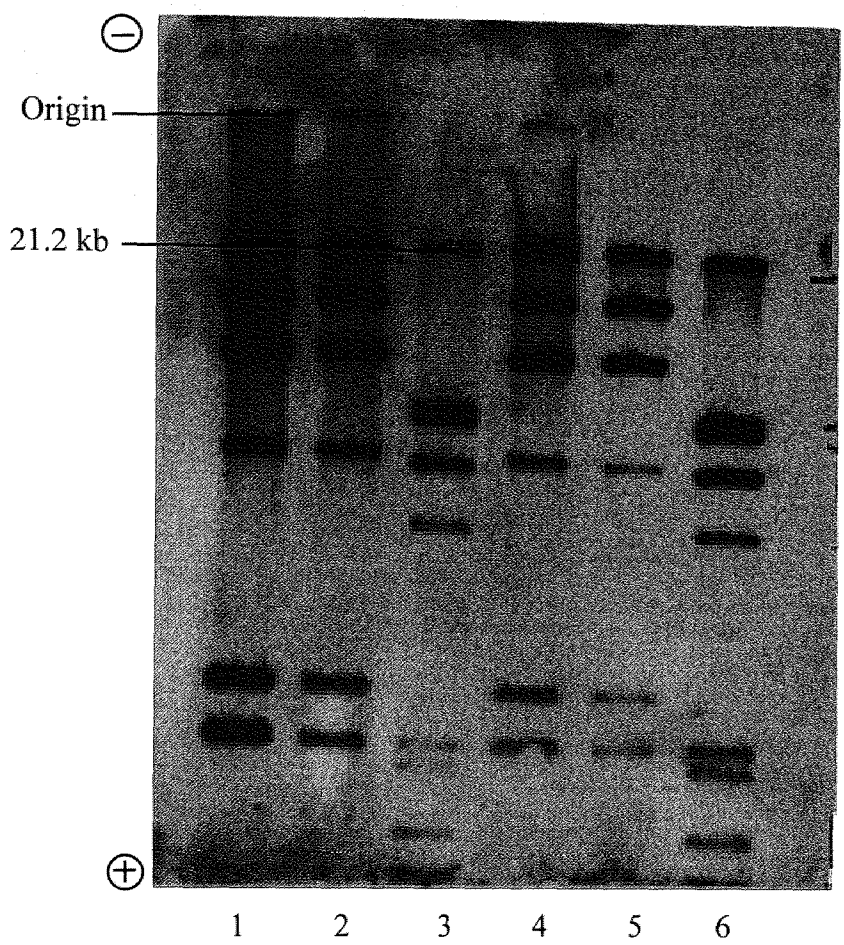

Fig. 8 Chemiluminescent mini-analytical gel film with digoxigenin labeled *Hind* III digested lambda DNA probe and Analytical Genetic Testing Center's (AGTC's) Uni-Block for prehybridization and immunological blocking reagents from 4-02-92: Figure 8 contains 6 lanes as follows: 1) 0.09 ug of *Hind* III digested lambda DNA, 2) 0.045 ug of *Hind* III digested lambda DNA, 3) 0.02 ug B-M MWM III Ladder, 4) 0.03 ug *Hind* III digested lambda DNA, 5) 0.015 ug *Hind* III digested lambda DNA, and 6) 0.01 ug B-M MWM III Ladder. Hybridization, electrophoresis, gel buffer, tank buffer and visualization parameters are the same as Figure 7. AGTC's Uni-Block was used for both prehybridization and immunological blocking.

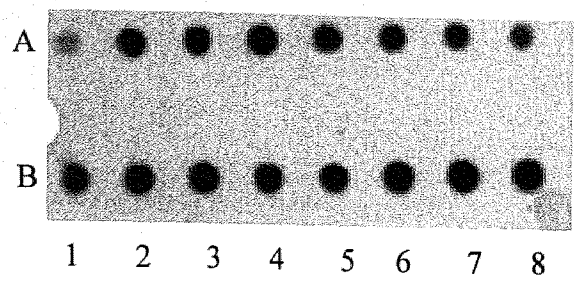

Fig. 9 Dot blot fixation membrane, air drying & U-V fixation vs heat bake and U-V fixation from 2-26-93: Row A contains 8 spots with 3.2 ng/spot of human DNA. Ultraviolet exposure, (254 nm), of the spots from left to right are 0 sec, 30 sec, 45 sec, 1.0 min, 2.0 min, 3.0 min, 4.0 min, and 5.0 min. This strip of membrane was air dried before ultraviolet fixation. Row B contains 8 spots with 3.2 ng/spot of human DNA. Ultraviolet exposure is the same as top row. This strip of membrane was heat baked at 80°C for 30 min before U-V fixation. Exposure was 1.0 hr with Lumi-Phos™ 530 on Kodak XAR x-ray film. Samples were applied with the BRL Convertible™ Filtration Manifold System, and developed with the Aces™ Human DNA Quantitation System.

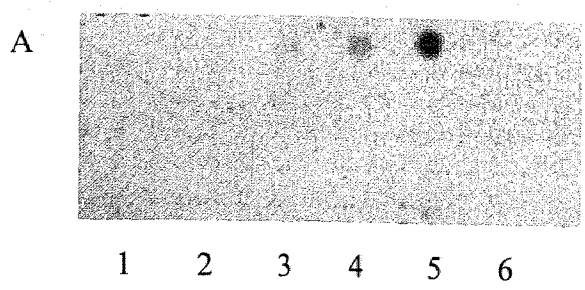

Fig. 10 Dot blot fixation membrane U-V exposure while damp: Row A contains 5 spots of human DNA. The sixth spot is a negative control. Spot concentrations are as follows: 1) 0.16 ng, 2) 0.32 ng, 3) 0.8 ng, 4) 1.6 ng, 5) 3.2 ng, and 6) 0 ng. This membrane was exposed to ultraviolet light at 254 nm for 1.0 minute while still damp to fix DNA to the membrane. Exposure was 1.0 hr with Lumi-Phos™ 530 on Kodak XAR x-ray film. Samples were applied with the BRL Convertible™ Filtration Manifold System and developed with the Aces™ Human DNA Quantitation System.

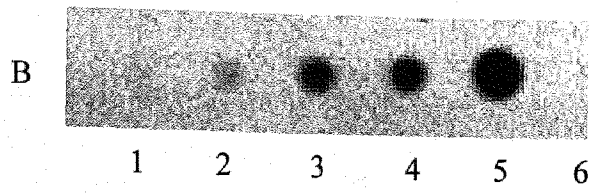

Fig. 11 Dot blot fixation membrane U-V exposure after drying from 2-26-93: Row B contains 5 spots of human DNA. The sixth spot is a negative control. Spot concentrations are exactly the same as figure 10. After air drying the membrane it was exposed to ultraviolet light at 254 nm for 1.0 min. Exposure with Lumi-Phos™ 530 and sample application were exactly the same as Figure 10.

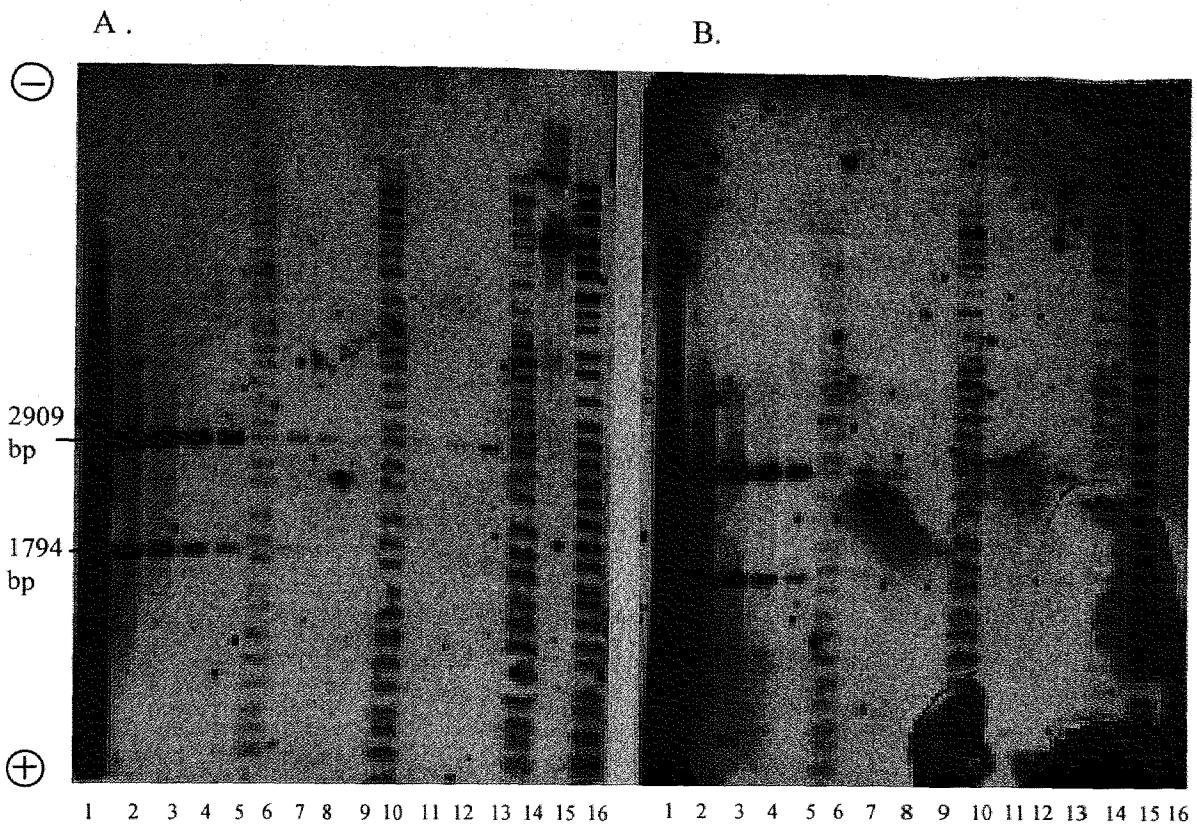

Fig. 12 Chemiluminescent analytical gel film YNH 24 probe 20 hr Lumi-Phos™ 530 exposure from 3-19-93: Film 12A contains 16 lanes as follows: 1) 120 ng lambda DNA *Hind* III digest, 2) 4.0 ug K562 human DNA, 3) 2.0 ug K562 human DNA, 4) 1.0 ug K562 human DNA, 5) 0.5 ug K562 human DNA, 6) 1.0 ng Gene Print™ Ladder, 7) 100 ng K562 human DNA, 8) 50 ng K562 human DNA, 9) 25 ng K562 human DNA, 10) 2.0 ng Gene Print™ Ladder, 11) 25 ng K562 human DNA, 12) 50 ng K562 human DNA, 13) 100 ng K562 human DNA, 14) Gene Print™ Ladder, 15) 105 ng lambda DNA, 16) 0.5 ul BRL Ladder. Film 12B contains 16 lanes as follows: 1) 120 ng lambda DNA *Hind* III digest, 2)

4.0 ug K562 human DNA, 3) 2.0 ug K562 human DNA, 4) 1.0 ug K562 human DNA, 5) 0.5 ug. K562 human DNA, 6) 1.0 ng. Gene-Print™ Ladder, 7) 100 ng. K562 human DNA, 8) 50 ng K562 human DNA, 9) 25 ng K562 human DNA, 10) 2.0 ng Gene Print™ Ladder, 11) 25 ng K562 human DNA, 12) 50 ng K562 human DNA, 13) 100 ng K562 human DNA, 14) 1.0 ng Gene Print™ Ladder, 15) 1.5 ul BRL Ladder, 16) 105 ng lambda DNA. All human K562 DNA samples have been digested with *Hae* III. Electrophoresis was at 32 volts for 16 hrs. Gel was 1% agarose in 1X TAE buffer. Tank buffer was same as gel buffer. Hybridization was 45 min at 55°C with fresh A-P labeled YNH24 probe. Visualization was with Lumi-Phos™ 530 for 20 hrs using Kodak XAR X-ray film.

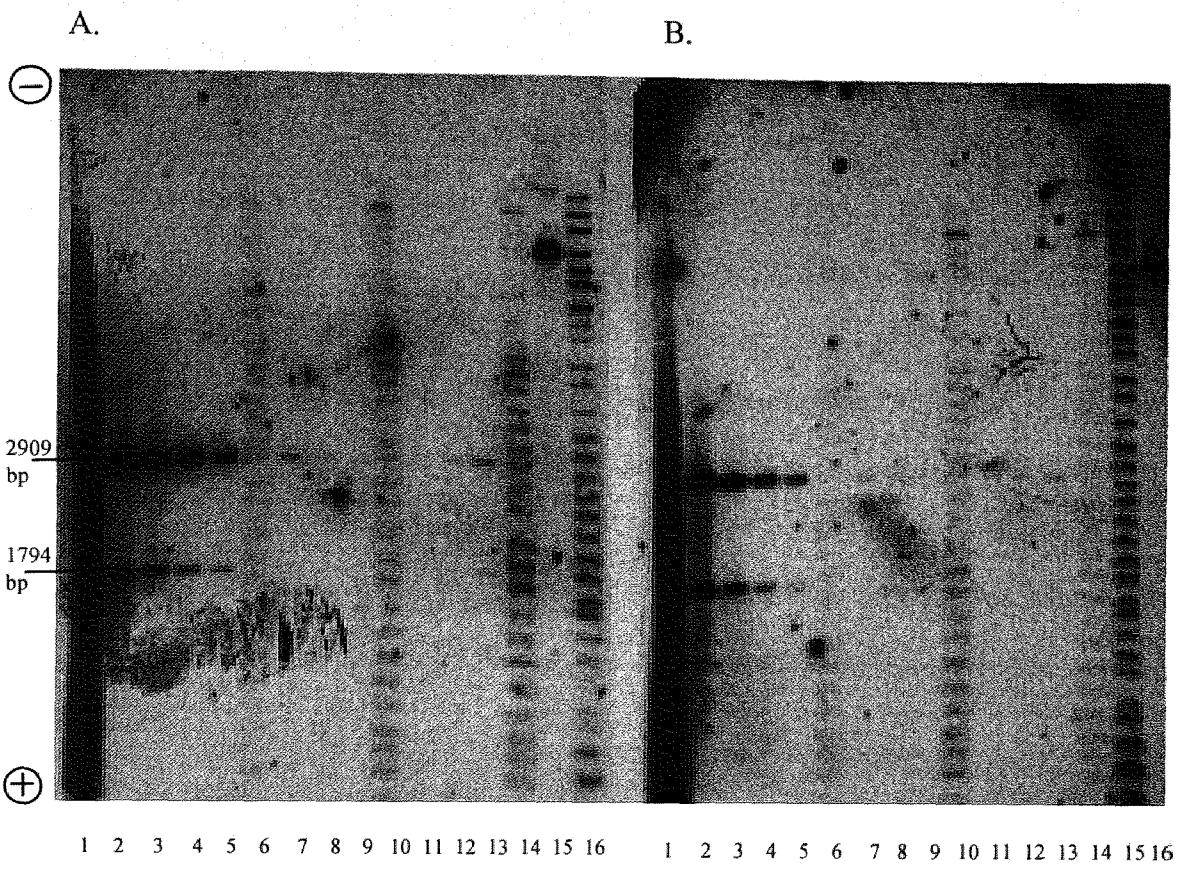

Fig. 13 Chemiluminescent analytical gel film YNH24 probe 7.0 hr Lumi-Phos™ 530 exposure 13A with mirror, 13B without mirror from 3-19-93: Film 13A contains 16 lanes as follows: 1) 120 ng lambda DNA *Hind* III digest, 2) 4.0 ug K562 human DNA, 3) 2.0 ug K562 human DNA, 4) 1.0 ug human DNA, 5) 0.5 ug K562 human DNA, 6) 1.0 ng Gene Print™ ladder, 7) 100 ng K562 human DNA, 8) 50 ng K562 human DNA, 9) 25 ng K562 human DNA, 10) 2.0 ng Gene Print™ ladder, 11) 25 ng K562 human DNA, 12) 50 ng K562 human DNA, 13) 100 ng K562 human DNA, 14) 2.0 ng Gene Print™ ladder, 15) 105

ng lambda DNA, 16) 0.5 ul BRL ladder. Film 13B contains 16 lanes as follows: 1) 120 ng lambda DNA *Hind* III digest, 2) 4.0 ug K562 human DNA, 3) 2.0 ug K562 human DNA, 4) 1.0 ug K562 human DNA, 5) 0.5 ug K562 human DNA, 6) 1.0 ng Gene Print™ ladder, 7) 100 ng K562 human DNA, 8) 50 ng K562 human DNA, 9) 25 ng K562 human DNA, 10) 2.0 ng Gene Print™ ladder, 11) 25 ng K562 human DNA, 12) 50 ng K562 human DNA, 13) 100 ng K562 human DNA, 14) 1.0 ng Gene print™ ladder, 15) 1.5 ul BRL ladder, 16) 105 ng lambda DNA. All human K562 DNA samples have been digested with *Hae* III. Electrophoresis was at 32 volts for 16 hrs. Gel was 1% agarose in 1X TAE buffer. Tank buffer was the same as gel buffer. Hybridization was for 45 minutes at 55°C using fresh A-P labeled YNH24 probe. Exposure was with Lumi-Phos™ 530 for 7.0 hrs with Kodak XAR X-ray film. Film 13A has been sandwiched between 2 mirrors for the 7.0 hr exposure time. Film 13B has not.

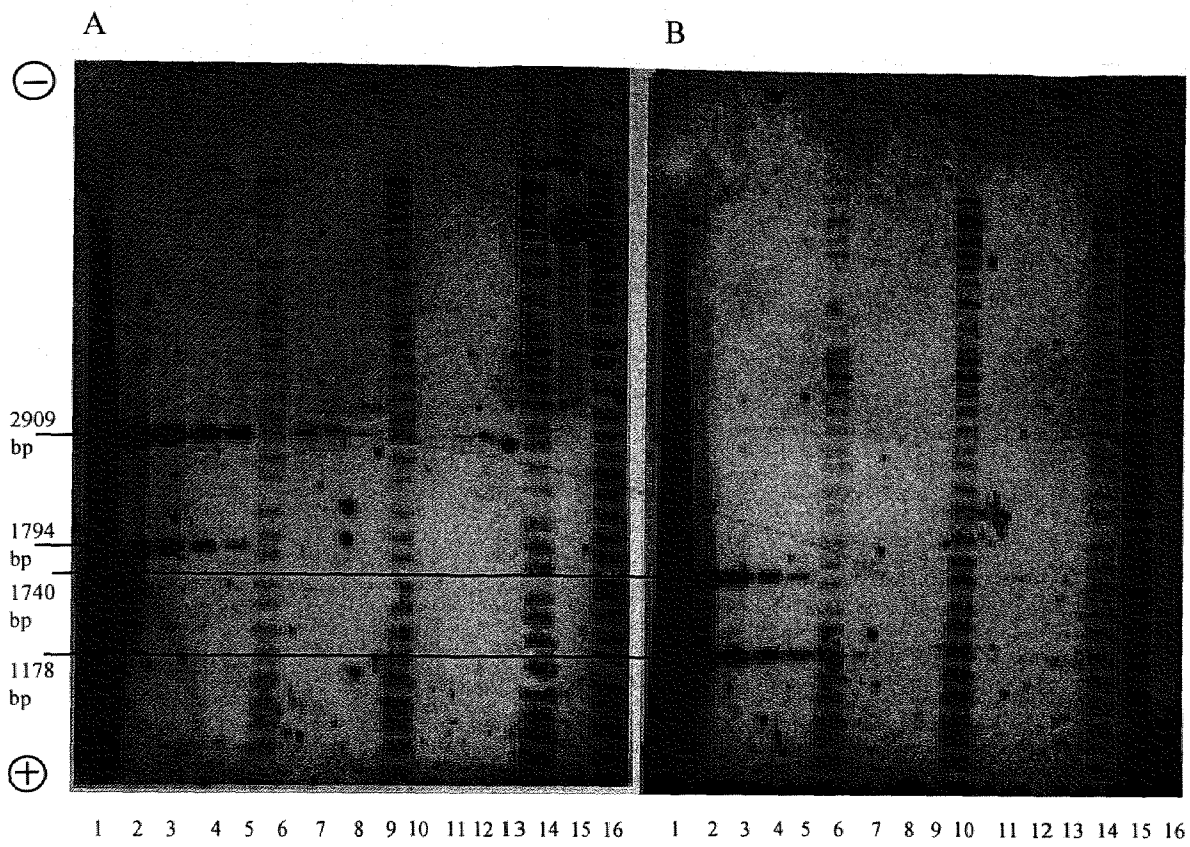

Fig 14 Chemiluminescent analytical gel film 14A rehybridization with YNH24 probe, 14B rehybridization with TBQ7 probe, 23 hr Lumi-Phos™ 530 exposure from 3-18-93: Film 14A contains 16 lanes exactly as figure 12A. Film 14B contains 16 lanes exactly as figure 12B. Electrophoresis, gel buffer, and tank buffer parameters were the same as from figure 12. Old probe was stripped off according to Promega Gene Print Light™ protocol. Rehybridization of the membranes with A-P labeled YNH24 probe on membrane 14A, and with A-P labeled TBQ7 probe on 14B took place for 45 min at 55°C. Exposure was with Lumi-Phos™ 530 for 23 hrs on Kodak XAR X-ray film.

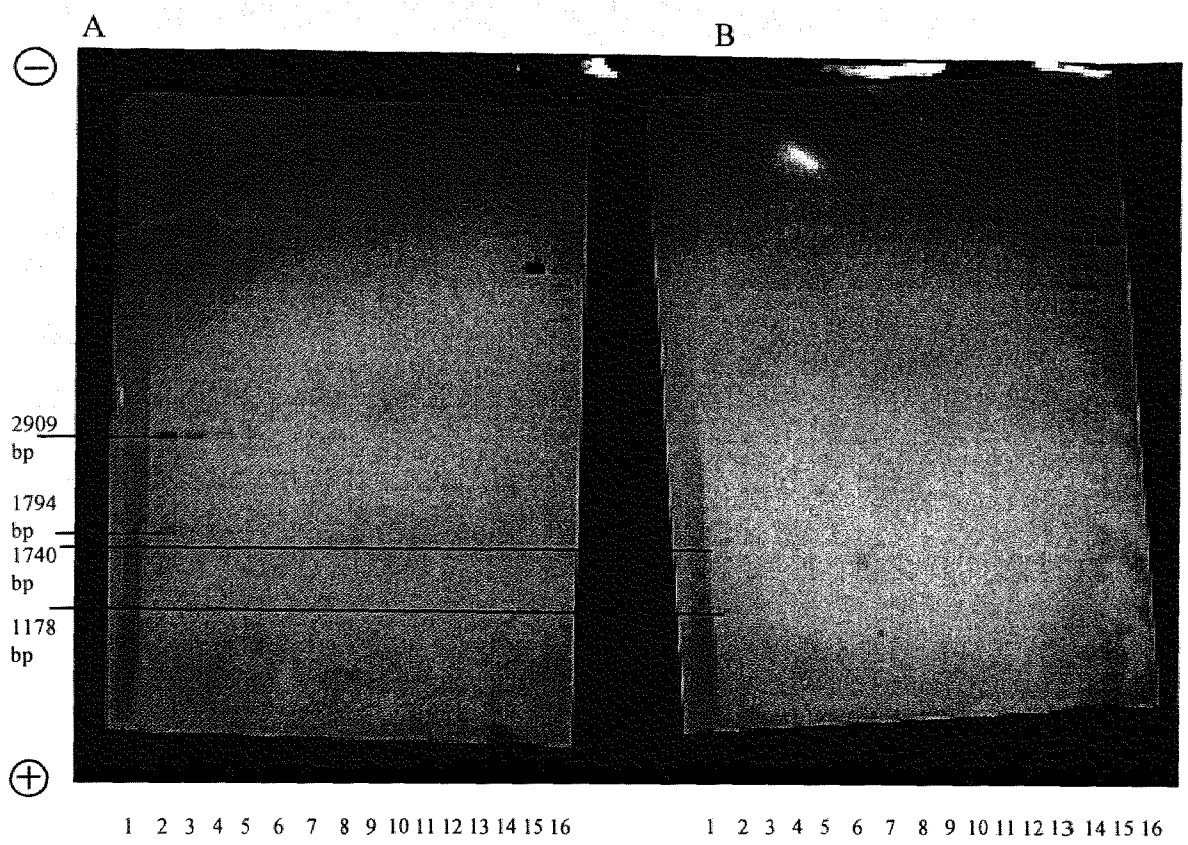

Fig 15 Colorimetric analytical gel membrane, 15A rehybridization with YNH24 probe, 15B rehybridization with TBQ7 probe, 24 hr NBT-BCIP development from 3-24-93. Membrane 15A contains 16 lanes exactly as figure 12A. Membrane 15B contains 16 lanes exactly as figure 12B. Electrophoresis, gel buffer and tank buffer parameters were the same as Figure 12. Old probe was stripped off according to Promega Gene Print Light™ protocol. Rehybridization of the membranes with A-P labeled YNH24 probe on membrane 15A, and with A-P labeled TBQ7 probe on membrane 15B took place for 45 min at 55°C. Visualization was with NBT-BCIP colorimetric development for 24 hrs.

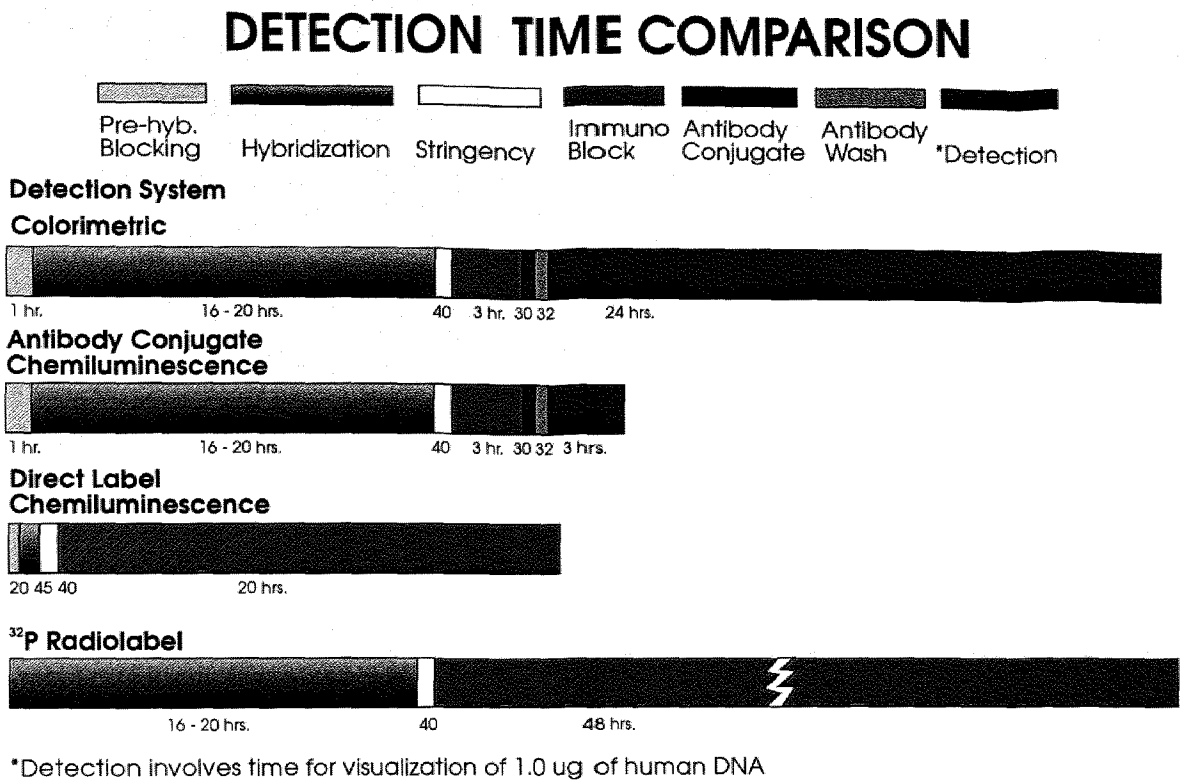

Chart 1 Bar chart time comparison of four different detection systems for DNA RFLP analysis. Times are listed in hours; however, times without the hrs designation are in minutes. (Note: Time bar chart for ^{32}P is based on FBI protocol's published results.)

A comparison of detection times between the four systems showed considerable variation. Direct labeled chemiluminescence was the shortest with detection times under 22 hrs. The double antibody chemiluminescence was next with detection times in the 25-29 hr range. This was based on an extended overnight hybridization of 16-20 hrs with extended blocking times and Lumi-Phos™ 530 exposure. This accounts for the discrepancy in their 17-21 hr procedure time table as listed by the manufacturer. The colorimetric procedure came in third with detection times of 46-50 hrs using the Boehringer Mannheim antibody conjugate labeling system and NBT-BCIP for visualization. This system could be shortened to the same time frame of the direct label probe system with a substitution of the NBT-BCIP for Lumi-Phos™ 530. The radioactive ^{32}P system was the most time consuming with detection times of 65-69 hours. Another time consideration with using ^{32}P detection is the time needed in monitoring for radioactive contamination and record keeping of the radioactive materials for both storage and disposal. Licensing with the NRC can be done at the state level through the State Department of Health.

Cost comparison of the three systems showed a considerable difference of hardware and technician time. The comparison of these was based on items used specifically for this method. Items that were used across the board for all methods were not included. An estimate was based on the use of 100 11x15 cm membranes.

HARDWARE DETECTION COST COMPARISON

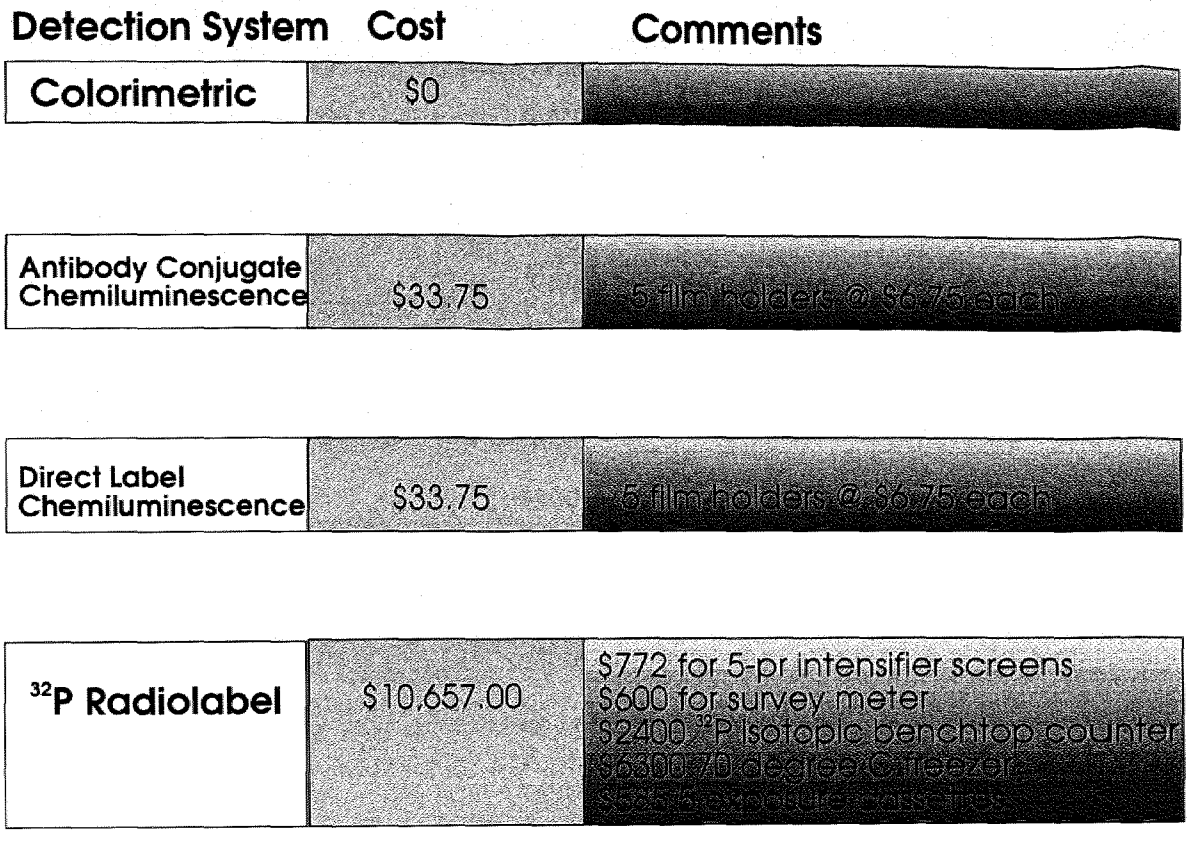

Chart 2 Hardware cost comparison of four different detection systems for DNA RFLP analysis. Costs include major hardware needed to perform tests.

(Note: Cost graph for ^{32}P is based on the FBI Protocol's published information.)

CONSUMABLES DETECTION COST COMPARISON

Supplies	Colorimetric	Double Ab Chemilumines	Direct Label Chemilumines	^{32}P Radiolabel
2 - Genius kits	$680	$680		
Probe	*$1725 1500 ng (pH 30)	*$1725 1500 ng (pH 30)	*$600 2.4ml Probe YNH-24	Probe (pH30) with BRL Ladder $748 $680 17 Shipments
Ladder	*$255 B-M MWM #III	*$255 B-M MWM #III	*$400 800 ng Equiladder	
20 mg Glycogen	$54	$54		
180mg Salmon Testes DNA	$180	$180		
150 ml Lumiphos		$390	$390	
X-Ray Film & Chemistry		$148	$148	$148
Total	$2894	$3432	$1538	$1576

*Note Digoxigennin labeled probes and ladders, along with Direct Link A-P labeled probes and ladders may be re-used multiple times therefor reducing cost 50-75% on these reagents.

Chart 3 Consumables cost comparison of four different detection systems for DNA RFLP analysis. Prices are based on 100 11 x 15 cm membranes.

(Note: The detection cost for ^{32}P is based on the FBI Protocol's published data.)

From a hardware standpoint, cost is highest for the use of a ^{32}P radioactive detection system. A need for radioactive monitoring of probe requires an isotopic bench top counter to measure ^{32}P incorporation into the probe after labeling.. A survey meter is also needed to monitor contamination in the work area. The use of exposure cassettes with intensifier screens and therefore a -70°C freezer is needed in isotopic detection.

The cost factor of the Promega probes was based on research work. If the probe was to be used on case work, a standing agreement would have to be signed to pay royalties for use of the probes. This is a difference from the probes' high cost by Cellmark, Lifecodes, or GIBCO BRL who have a high initial cost but do not require royalties.

From a consumables standpoint, based on 100 11 x 15 cm membranes, the cost breakdown is actually not that different. Direct link chemiluminescence and radioactive ^{32}P detection are very close in price at $1538 vs. $1576. Please note, the cost of the direct link chemiluminescent probes were based on the research and development price of the probe from Promega. Once case work is begun, royalties will be paid to the company based on number of samples run. At this time no estimate can be made on that number so no estimate can be made on the actual cost of this probe. The colorimetric and double antibody chemiluminescence with Lumi-Phos™ 530 were higher in cost; however, since one can reuse the labeled probe repeatedly, the actual cost of these systems would be considerably cheaper since probe cost is the most expensive consumable. If one were to use these probes over 2-4 times the cost of these two systems would be reduced from the $2894 for colorimetric and $3432 for antibody conjugate chemiluminescence to $1744 and $2282 respectively.

From an ease of operation standpoint, The direct link probes with either the Lumi-Phos™ 530 or NBT-BCIP detection are easier to use than probes detected with the double antibody method of the Genius™ kits. This is based upon the fact that fewer steps are involved and therefore fewer reagents. Background is also less of a problem. One added

advantage of Lumi-Phos™ 530 exposure is that films can be placed on both sides of the membrane to get two different timed exposures. Films can then be batch processed for dark room development at a later time just as with radioisotopic detection. Probe stripping is quite easy with direct label probes since only sodium dodecyl sulfate is used at high temperature. Dimethyl formamide for probe stripping in either the Genius™ kits or FBI radioactive protocol is dangerous since its flash point is 67°C. Included in the ease of operation is safety, which is much more stringent with the radioisotopic method. Each item that comes in contact with radioisotopic ^{32}P, must be monitored for contamination. This is a significant time consuming process for probe stripping, hybridizations, and probe labeling. Also stringent record keeping must be kept to monitor incoming and out going radioisotopes. This includes dumping of liquid waste down the drain and storing hot solid waste in short term storage for 10 times its half-life. More instrumentation is also required for the ^{32}P detection, which includes a -70°C freezer, survey meter, and scintillation counter,

From a sensitivity stand point, ^{32}P is still the most sensitive detection technique (7, 24), showing detection limits to 20 ng (Personal communication Howard Coleman Genelex) of DNA to get RFLP results. Promega claims 50 ng detection with their chemiluminescence system. Results did not show this degree of sensitivity. A more reasonable detection limit of 100 ng was demonstrated. Background still was a problem; however, with more practice results should improve. The colorimetric detection of a direct link probed membrane showed a 500 ng detection limit after 24 hr incubation with NBT-BCIP. The results were easily read and little background was present. The double antibody chemiluminescent detection method using the Boehringer Mannheim kit and pH30 probe only showed a detection limit of 2.0 ug; however, this was using a 1.0 hr exposure, and with non-optimum probe labeling and detection methods. Background was still the most significant problem in this system. This is an inherent problem in any double antibody system such as digoxigenin or

biotin due to nonspecific binding of the DNA to the membranes. That is why so much time was spent working with different blocking reagents and the addition of salmon testes DNA to help block unbound sites on the membrane. The actual sensitivity of the double antibody system was not determined. Money and reagents ran out. A different probe should have been chosen or a different labeling strategy for that specific probe should have been used. All of this information is summarized in table 4. Currently, there are more labeling techniques for probes available through Boehringer Mannheim than were available when this project was started.

Statistical assessment of the three systems detection limits was not possible since the double antibody chemiluminescent system did not work, and the DCI laboratory is not running ^{32}P isotopic detection.

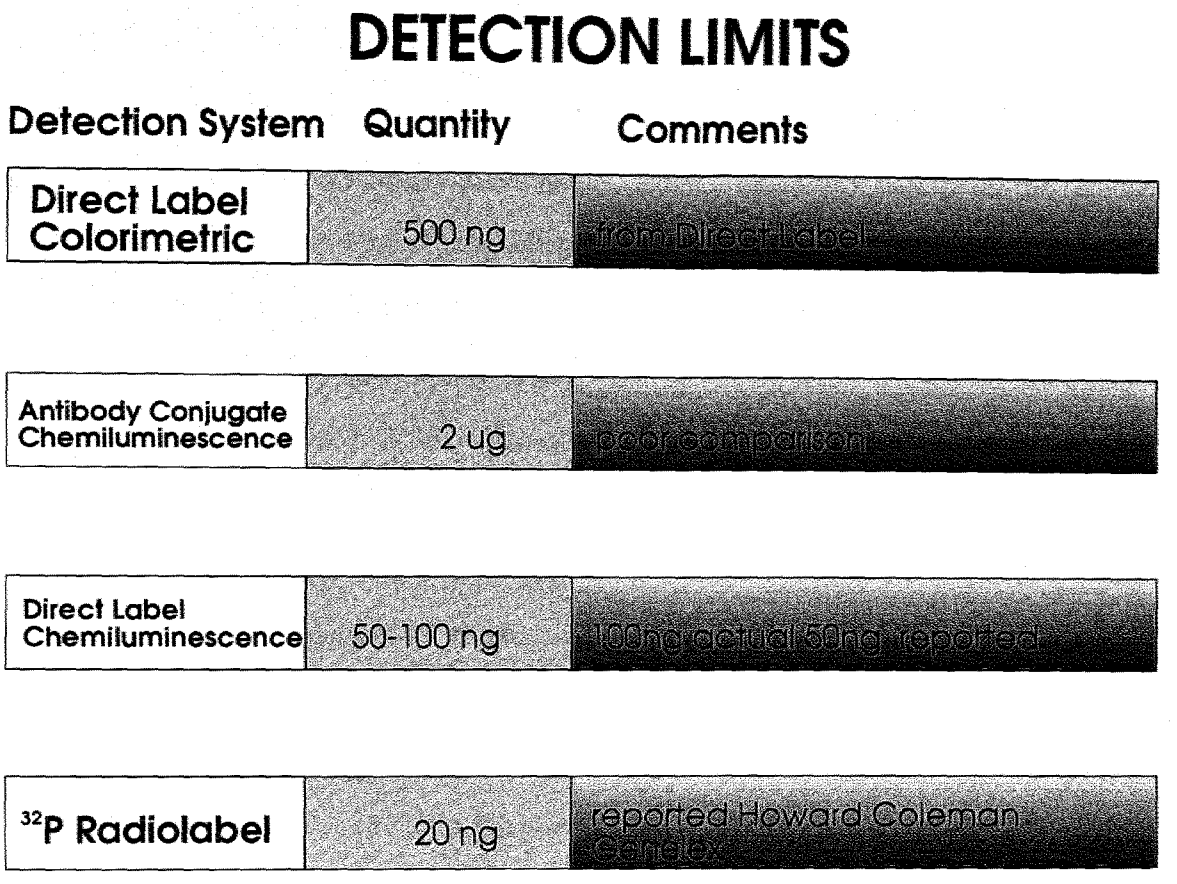

Chart 4 Comparison of the detection limits of four different detection systems. Work is based on DNA RFLP analysis. (Note: ^{32}P detection limit was reported from the literature, and was not derived empirically.)

CONCLUSIONS

From a cost, ease of operation, and time stand point, the direct link alkaline phosphatase labeled probes are clearly easier to use. From a sensitivity standpoint the radioactive ^{32}P is still 2-5 times more sensitive than by my work; however, work by GIBCO BRL has shown greater improvement in sensitivity. Papers presented at the Third International Symposium on Forensic DNA Analysis in Quantico, VA by GIBCO BRL in March 1993 reported that the optimized ACES™ II system had sensitivities greater or equal to that of ^{32}P detection. With GIBCO BRL marketing probes D1S7 and D4S139, and Promega marketing probes D2S44 and D10S28, all of which are direct link alkaline phosphatase labeled probes, a reasonable number of probes are available for forensic DNA RFLP analyses. Whether or not they are used for small stains where less than 50 ng of DNA are detected, they certainly warrant use in data base work. Also because of the increased interest in polymerase chain reaction (PCR), mitochondrial DNA, amplified fragment length polymorphism (AMFLP), and short tandem repeats (STRs) , a reasonable stain size could be assessed and the more sensitive tests could be run after determination of the quantity and quality of DNA present.

Since the start of this project laboratories are coming on line with a polymerase chain reaction based (HLA DQ alpha) test and an amplified fragment length polymorphism based (D1S80) test. Both of these testing procedures are manufactured in kit form and use a thermocycler to amplify the DNA. Those laboratories using them over the next several years will most likely determine the direction of forensic DNA testing; however, the judicial system and U.S. Congress will also have an impact concerning acceptability in the courts, quality control of the tests, proficiency monitoring and funding.

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APPENDIX

Boehringer Mannheim Technical Bulletins:

- •Technical Update: Genius™ Nonradioactive DNA Labeling and Detection Kits, July 91.
- •DIG DNA Labeling and Detection Kit Non radioactive, Aug. 90.
- •Lumi-Phos™ Chemiluminescent formulation for alkaline phosphatase detection when using the Genius™ Nonradioactive Nucleic Acid labeling and Detection System 530, June 91.
- •Technical Update DIG-System.
- •Boehringer Mannheim, "Genius™ Nonradioactive DNA Labeling and Detection Kit Technical Update," July 1991.
- •Boehringer Mannheim, "DIG DNA Labeling and Detection Kit Nonradioactive," Catalog Number 1093 657, Revised Aug. 1990.
- •Boehringer Mannheim, "Lumi-Phos™ 530," Catalog Number 1275 470, June 1991.

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Promega Technical Manual Gene Print Light™ DNA Typing Products Chemiluminescent Detection Part #TM002, Revised 8/92.

The 1992/93 Promega DNA Typing Catalogue #CA D002 Promega Corporation 2800 Woods Hollow Road, Madison, WI 53711-5539.

ACES™ Human DNA Quantitation System Catalogue No. 4220SA, GIBCO BRL Life Technologies Inc., Aug 5, 1992.

The Convertible™ Filtration Manifold System Cat. Series 1055, Instruction Manual, GIBCO BRL Life Technologies Inc.

Kodak Chemical Inserts:

Kodak GBX developer and Replenisher

Kodak GBX Fixer and Replenisher

Kodak X-OMAT AR film

Reagents

Boehringer Mannheim

Genius™ Nonradioactive DNA Labeling and Detection Kits

Lumi-Phos™ 530

DNA molecular weight marker III., Digoxigenin-labeled

Glycogen 20 mg/ml

Polaroid

Polaroid #667 High Speed Black and White film

Sigma

Protease K, 12.3 units/mg solid

Lambda DNA-*Hind* III digested

Agarose Molecular Biology Grade (eeo 0.15) A9539

Lauryl Sulfate Sodium Salt (SDS) L4390

Phenol: Chloroform: Isoamyl Alcohol

Kodak X-OMAT AR (XAR-5) 8"x10" film

Kodak GBX Fixer and Replenisher

Kodak GBX Developer and Replenisher

Quick Draw Blot Paper 20 x 20 cm P8171

Nylon membranes 20 x 20 cm

Whatman

3mm chromatography paper

Schleicher and Schuell

Blot pads 11x14 cm

GIBCO BRL Life Technologies Inc.

DNA Probe pH30 (D4S139)

DNA quantitation Standards

DNA K562 Typing Grade

Hae III restriction enzyme

Ladder ACES™ 30 band

Human DNA Quantitation System ACES™ #4220SA

AGTC Analytical Genetic Testing Center

Uni-Block

Kodak

Ethidium bromide

Micron Separations Inc.

MSI Magnagraph 20 x20 cm nylon membranes

MSI Magnagraph 15 cm x 3.0 m roll

MSI 0.22 um filtering units

Baker

Glycerol

Fisher

Bromophenol blue

Polyethylene glycol

LKB-Pharmacia

Repel silane

Promega

DNA Probe YNH 24

DNA Probe TBQ7

Gene Print Light™ Ladder

Hybridization Solution

DNA K562 *Hae* III digested

Equipment

Savant Micro centrifuge HSC 10K

Savant Speedvac Evaporator SC100

Savant Refrigerated Condensation Trap RT100

Savant Two Stage Vacuum Pump VP100

Fotodyne mini-gel electrophoresis tanks 7.1x9.3 cm

Fotodyne power supply Model 255

Fotodyne 302 nm Foto/Phoresis I U-V viewing box

Fotodyne Polaroid™ back camera Model FCR-10

Pharmacia small format electrophoresis tank GNA 100

Pharmacia large format electrophoresis tank 20 x 20 cm GNA 200

Fisher 312 nm U-V viewing box #FBTIV-88

Precision Model 19 Vacuum Drying Oven

Precision Gravity Convection Incubator [Hybridization oven

(with homemade rotisserie)]

Pipettors Sealpette brand 0.5-10, 2-20, 20-200, 200-1000 ul

Fisher Scientific Rotator

Hybaid mini oven MKII rotating hybridization oven

Hybaid mini-oven hybridization bottles: 30 cm x 3.5 cm, and 15 cm x 3.5 cm

Hermle z230 Microcentrifuge

Corning pH/Ion meter 150

Thelco Precision Model 83 water bath

Precision Scientific Model 25 Reciprocal Shaking water bath

Perkin Elmer U-V Visible spectrophotometer Lambda 3 with super sipper

GIBCO BRL Model H5 horizontal electrophoresis tanks 11x16 cm

GIBCO BRL Model 500 High Current Power Supply

Boiling microcentrifuge tube holder home made

Fisher heat block dry bath incubators

Barnstead C57835 Sterilizer

Fisher Biotech Ultraviolet Crosslinker FBUVXL 1000

GIBCO BRL The Convertible™ Filtration Manifold System #1055 dot blot

apparatus

Gast Model DOA-P104-AA vacuum pump